Screening of $IFN\gamma$ CA repeats and FOKI polymorphisms at the VDR gene in tuberculosis affected population from orissa.

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Executive Summary

Tuberculosis is an infectious disease known to persist latently within organisms, with many environmental, and genetic factors determining the progression of the infection. Additionally, weakened immune system which is a hallmark of diabetes mellitus, silicosis, leukaemia, kidney disease, corticosteroid, rheumatoid arthritis, and/or crohn's disease may also succumb to tuberculosis, thus highlighting the challenge for effective diagnosis, and prognosis. Interferonγ, and vitamin D3 receptor represent some of the paradoxical factors influencing the progression of tuberculosis within certain populations, but being uninformative in certain others. For example, the T allele of FokI RFLP is less frequently associated with Africans, and more frequently with Caucasians, and Asians. The focus of this project was to elucidate, and explore the variability of inter individual susceptibility and/or resistance responses to tuberculosis by conducting a case control association test through the candidate gene approach. Since the mutations within these two genes have been previously documented to increase susceptibility towards the mycobacterial infections, hence a case control approach was designed for the population of Orissa, and Chhattisgarh. Screening of variants was conducted by designing an RFLP for VDR, and SSCP for IFNγ. Through the preliminary results indicated the absence of a linear trend between the chosen categories; more data would be needed to conclusively prove the results.

Acknowledgements

I would like to start by thanking the archive organisation for providing avenues for creating a platform for thesis collection. Special thank you to the GNU, and LaTeX group for their continuous effort for development of the ever expanding typesetting language. Their methods, by far exceeds all devised so far specially with regard to the treatment of the white space, and the application of backslash.

History

I. Screening of CA repeats of IFNγ and FOKI polymorphism of VDR gene in tuberculosis affected population of orissa. - Thesis submitted to Jamia Millia Islamia (JMI) to obtain the degree of Master of Science (Biotechnology) in october, 2009.
II. Screening of IFNγ CA repeat and VDR FOKI polymorphisms in tuberculosis patients from orissa. - Thesis submitted to Jawaharlal Nehru University (JNU), in partial fulfillment for the requirement of Masters of Science (Biotechnology) in june, 2009.

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Abbreviations

Ags	Antigens
AFB	Acid fast bacteria
AMP	Anti microbial peptides
ARTI	Annual risk of tuberculosis infection
BCG	Bacille calmetter guérin
BMD	Bone mass density
CBH	Cutaneous basophils hypersensitivity
CD	Cluster of differentiation
CF	Cord factor
CNPs	Copy number polymorphisms
CTL	Cytotoxic T lymphocyte
DALYs	Disability adjusted life years
DOTs	Directly observed treatment short
HLA	human leukocyte antigen
HSC	Hematopoietic stem cells
IFN	Interferon
IL	Interleukin
INH	Iso nicotinyl hydrazide
iNOS	Inducible nictric oxide synthease
JAK	Janus kinase
LD	linkage disequilibrium
LJM	Lowenstein Jensen medium
LPS	lipo poly saccharide
MDP	Muramyl di peptide
MDR	Multi drug resistant
MHC	Major histocompatibility complex
NF	Necrosis factor
NO	Nitric oxide
NOD	Nucleotide oligomerization domain like receptors
Nramp	Natural resistance associated macrophage protein
NTP	National tuberculosis programme
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PLHAs	People living with HIV/AIDS
PPD	Purified protein derivative
PTH	Parathyroid hormone
RFLP	Restriction fragment length polymorphism
RNTCP	Revised national tuberculosis control programme
PRRs	Pattern recognition receptors
RXR	Retinoid X receptor

SCP	Start codon polymorphism
sHPT	secondary hyperparathyroidism parathyroid hormone
SSCP	Single strand conformational polymorphism
SNP	Single nucleotide polymorphism
SSLP	Single sequence length polymorphism
STAT	Signal transducers and activators of transcription
TB	Tuberculosis
TCR	Tool cell receptor
TH	T helper
TNF	Tumor necrosis factor
UTR	Untranslated region
VDR	Vitamin D3 receptor
VDREs	Vitamin D response elements
VNTR	Variable number tandem repeats
WHO	World health organisation
XDR	Extensively drug resistant

Part I

Introduction

1 Tuberculosis

Over a century ago Robert Koch identified Mycobacterium tuberculosis as the causative agent of tuberculosis (TB). At that time, TB was rampant, causing 1/7 of all deaths in Europe and 1/3 of deaths among productive young adults. Today, TB remains a global health problem of enormous dimension. Furthermore, because of the spread of AIDS and non compliance in the drug treatment, Mycobacterium tuberculosis is increasing in frequency once again and is becoming even more drug resistant. It is estimated that there are one billion people infected worldwide that is twenty percent of the world's human population, with ten million new cases and over three million deaths per year. Anything that can be learnt from genome studies could be of great importance in the fight to control the renewed spread of tuberculosis.

1.1 Alternative Names

- Consumption (because it seemed to consume people from within as people suffered from bloody cough, fever, pallor and long restless wasting).
- Phthisis or Phthisis pulmonalis (Phthisis is Greek for consumption where as Phthisis pulmonalis represents consumption of lungs)
- Scrofula (affecting the lymphatic system and resulting in swollen neck glands)
- Tabes mesenterica (tuberculosis of the abdomen)
- Lupus Vulgaris (tuberculosis of the skin)
- Wasting Disease or White Plague (because sufferers appear markedly pale)
- King's Evil (as it was believed that a king's touch would heal scrofula)
- Pott's Disease or Gibbus of the spine and joints (named after Percivall Pott and Gibbus because it involves skeletal deformities)

Although Mycobacterium tuberculosis is known as the principal cause of tuberculosis in humans but certain other mycobacteria present in Mycobacterium Tuberculosis Complex and Non-tubercolous bacteria are also known to cause TB. The Mycobacterium Tuberculosis complex includes other TB causing mycobacteria.

Mycobacterium bovis was once a major cause of tuberculosis, but the introduction of pasteurized milk has largely eliminated this as a public health problem in developed countries.

Mycobacterium africanum is not widespread, but in parts of Africa it is a significant cause of tuberculosis. Mycobacterium microti is mostly seen in immunodeficient people.

Mycobacterium Canetti is a novel pathogenic taxon of the Mycobacterium tuberculosis complex. This strain was isolated from a 2-year-old Somali patient with lymphadenitis. It did not differ from Mycobacterium tuberculosis in the biochemical tests and in its 16S rRNA sequence, but formed smooth and shiny colonies, which is highly exceptional for this species. Additionally, it had shorter generation time than clinical isolates of M. tuberculosis and presented a unique, characteristic phenolic glycolipid and lipo-oligosaccharide. This compound was observed in a smooth isolate of M. tuberculosis described in a farmer by the French microbiologist Georges Canetti, after whom the organism has been named. Tuberculosis caused by M. Canetti appears to be an emerging disease in the Horn of Africa.

1.2 Non-tuberculosis bacteria

The Non-tuberculosis bacteria include the following bacteria.

Mycobacterium avium which is a member of Mycobacterium avium complex (MAC). MAC bacteria are common in the environment and cause infection when inhaled or swallowed. Symptoms of MAC diseases are reminiscent of tuberculosis. They include fever, fatigue, and weight loss. Many patients will have anaemia and neutropenia if bone marrow is involved. Pulmonary involvement is similar to TB, while diarrhoea and abdominal pain are associated with gastrointestinal involvement. MAC disease is common in immunocompromised individuals, including senior citizens and those suffering from HIV or cystic fibrosis; however, the MAC diseases, particularly Lady Winderemere syndrome, do not require that the individual be immunocompromised.

Mycobacterium kansasii The most common presentation of M kansasii infection is a chronic pulmonary infection that resembles pulmonary tuberculosis. However, it may also infect other organs. M kansasii infection is the second-most-common nontuberculous opportunistic mycobacterial infection associated with AIDS, surpassed only by Mycobacterium avium complex (MAC) infection. For this reason, the incidence of M kansasii infection has increased because of the HIV/AIDS epidemic.

The second edition of Bergey's manual classifies the actinomycetes and other high G+C gram positive bacteria using 16S rRNA data. Actinomycetes have considerable practical impact because they play a major role in the mineralization of organic matter in the soil and are the primary source of most naturally synthesized antibiotics. The genera Corynebacterium and Mycobacterium contain important human pathogens.

Firmicutes/ (low-G+C)	Bacilli	Lactobacillales (Cat-)	Streptococcus	Alpha hemolytic	optochin susceptible: *S. pneumoniae* (Pneumococcal infection) optochin resistant: *S. viridans (S. mitis, S. mutans, S. oralis, S. sanguinis, S. sobrinus, milleri group)*
				Beta hemolytic	A, bacitracin susceptible: *S. pyogenes* (Scarlet fever, Erysipelas, Rheumatic fever, Streptococcal pharyngitis) B, bacitracin resistant, CAMP test+: *S. agalactiae*
				Gamma hemolytic	D, BEA+: *Streptococcus bovis*
			Enterococcus	BEA+: *Enterococcus faecalis* · *Enterococcus faecium*	
		Bacillales (Cat+)	Staphylococcus	Cg+ *S. aureus* (Staphylococcal scalded skin syndrome, Toxic shock syndrome) Cg- *novobiocin susceptible* (*S. epidermidis*) · *novobiocin resistant* (*S. saprophyticus*)	
			Bacillus	*Bacillus anthracis* (Anthrax) · *Bacillus cereus* (Food poisoning)	
			Listeria	*Listeria monocytogenes* (Listeriosis)	
	Clostridia	*spore-forming, motile:* *Clostridium difficile* (Pseudomembranous colitis) · *Clostridium botulinum* (Botulism) · *Clostridium tetani* (Tetanus) *spore-forming, nonmotile:* *Clostridium perfringens* (Gas gangrene, Clostridial necrotizing enteritis) *non-spore forming:* *Peptostreptococcus*			
	Mollicutes	Mycoplasmataceae	*Ureaplasma urealyticum* (Ureaplasma infection) · *Mycoplasma genitalium* · *Mycoplasma pneumoniae* (Mycoplasma pneumonia)		
		Anaeroplasmatales	*Erysipelothrix rhusiopathiae* (Erysipeloid)		
Actinobacteria/ (high-G+C)	Actinomycineae	Actinomycetaceae	*Actinomyces israelii* (Actinomycosis/Actinomycetoma) · *Tropheryma whipplei* (Whipple's disease)		
		Propionibacteriaceae	*Propionibacterium acnes*		
	Corynebacterineae	Mycobacteriaceae	*M. tuberculosis/M. bovis*	Tuberculosis: Ghon focus/Ghon's complex · Pott disease · *brain* (Meningitis, Rich focus) · *cutaneous* Tuberculous pericarditis	
			M. leprae	Leprosy	
			Nontuberculous	R1: *M. kansasii* R3: *M. avium complex* (MAA, MAP, MAI, Lady Windermere syndrome) · *M. ulcerans* (Buruli ulcer)	
		Nocardiaceae	*Nocardia asteroides/Nocardia brasiliensis* (Nocardiosis, Maduromycosis, Mycetoma) · *Rhodococcus equi*		
		Corynebacteriaceae	*Corynebacterium diphtheriae* (Diphtheria) · *Corynebacterium minutissimum* (Erythrasma) · *Corynebacterium jeikeium*		
	Bifidobacteriaceae	*Gardnerella vaginalis*			

Table 1: Classification of Mycobacteria on the basis of high G+C.
‡ *Source : http : //en.wikipedia.org/wiki/File : Tuberculosis_symptoms.svg*

1.2.1 Mycobacterium species infecting human host

Group and Species	**Clinical significance**
Tuberculosis group M tuberculosis M bovis	 both species are pathogenic and cause tuberculosis, they regularly exhibit susceptibility to antituberculosis drugs.
Leprosy group M leprae	 causes leprosy, has never been cultivated on laboratory media; can be grown in mouse footpads or in armadillos, where the temperature is favourable for growth (2 to 50°C below that of most animals)

Table 2: Groups and Species of Mycobacterium along with their clinical significance.

Group and Species	Clinical significance
Runyon groups*	
I. Photochromogens nonpigmented when grown in dark; Yellow pigment formed when grown in the light slow growing; M kansasii M marinum	 causes tuberculosis like disease. skin papules and ulcers; contracted from swimming in fresh or salt water; prefers temperature of 31°C for growth.
II. Scotochromogens red-orange pigment formed when grown in dark or light; slow growing M scrofulaceum M szulgai	 causes cervical adenitis in young children. pulmonary disease, adenitis, bursitis.
III. Nonchromogens nonpigmented in dark or light; slow growing. M avium/M intracellulare group M xenopi M ulcerans	 tuberculosis like disease in adults; lymphadenitis in children, usually resistant to ordinary antituberculosis drugs. tuberculosis like disease. ulceration and necrosis of skin.
IV. Rapid growing may be pigmented or non pigmented M fortuitum M chelonei	 local abscess at the site of a trauma; occasionally causes a tuberculosis like disease. has been isolated from patients with chronic respiratory disease.

Table 3: Runyon groups classify mycobacteria into Photochromogens, Scotochromogens, Nonchromogens and Rapid growing pigmented or non pigmented.

1.2.1.1 Classification

The following is the complete taxonomic classification for the species Mycobacterium tuberculosis as stated in Bergey's Manual of Systematic Bacteriology 2nd editiob, volume one.
Domain: Bacteria (that possess cell wall and lack nucleus and are prokaryotic)
Phylum: Actinobacteria (high G + C content in their DNA)
Class: Actinobacteria (high G + C content in their DNA)
Subclass: Actinobacteridae (rigid bacilli or filamentous cells that have a tendency to branch)
Order: Actinomycetales (rigid bacilli or filamentous cells that have a tendency to branch)
Suborder: Corynebacterineae (in addition to having a Gram-positive type cell wall, form metachromatic granules and their walls have meso-diaminopimelic acid.)
Family: Mycobacteriaceae (these bacterial cells typically do not branch, so any mycelium formation tends to be undeveloped. Moreover they are aerobic organisms that parasitize both humans and animals)
Genus: Mycobacterium (catalase positive, can form filaments which are readily fragmented, walls have high lipid content)
Species: Mycobacterium tuberculosis

1.3 Epidemiology of Tuberculosis

NATIONAL STATUS : India has the highest TB burden accounting for one fifth of the global incidence (Global annual incidence estimate is 9.1 million cases out of which it is estimated that 1.9 million cases are from India). India is 17th among 22 High Burden Countries in terms of TB incidence rate (Source: WHO global TB report 2008). Nearly 40 % of the Indian population is infected with the TB bacillus. Each year, 1.9 million new cases of TB occur in the country, of which about 0.8 million are infectious new smear positive pulmonary TB cases. The national ARTI (Annual Risk of TB infection) was estimated at 1.5% i.e. 75 new smear positive pulmonary TB cases are expected per 100,000 population annually. By any measure the burden of TB in India is staggering. More than 80% of the burden of tuberculosis is due to premature death, as measured in terms of disability-adjusted life years (DALYs) lost. Every day, more than 5,000 people develop TB disease, and nearly 1,000 people die of TB, i.e. 2 deaths every 3 minutes. As per WHO estimates in 2006, nearly 322,000 persons in India died of tuberculosis (mortality rate 28 per 100,000 persons), which was estimated at over 500,000 annually at the beginning of the revised national TB control programme (RNTCP). Deaths due to TB exceed the combined deaths from

all other communicable diseases and account for 26% of all avoidable adult deaths. TB is also the leading killer of women, causing more orphans than those produced by all causes of maternal mortality combined.

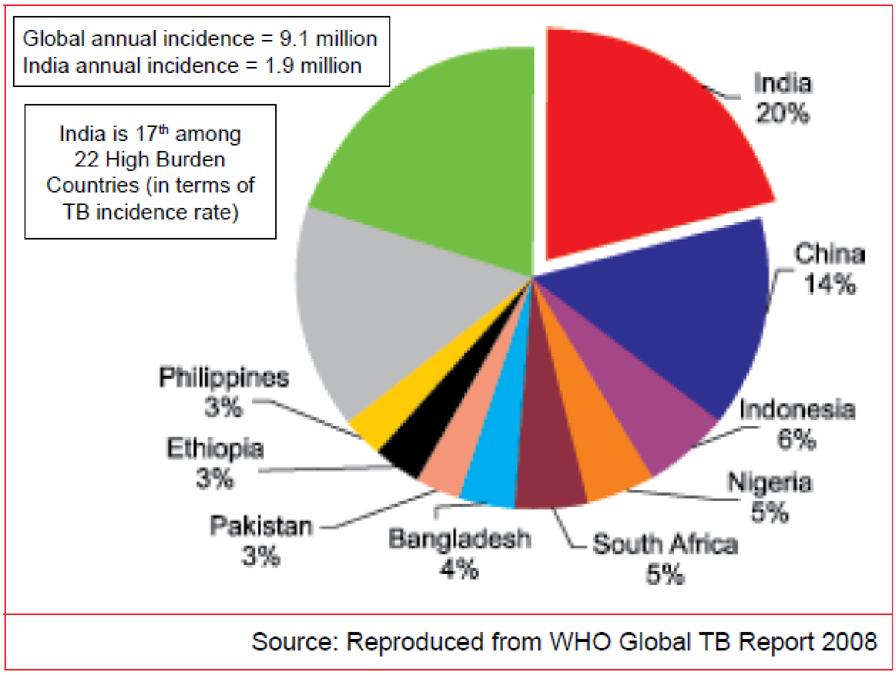


Figure 1: India as the highest TB burden country.

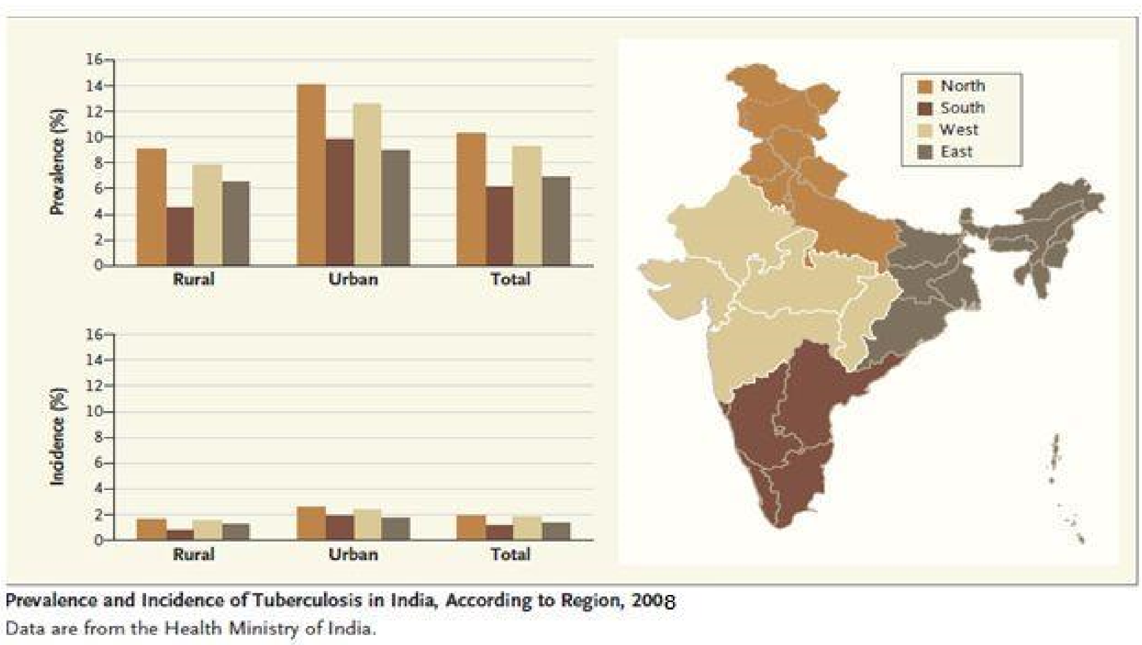


Figure 2: Prevalence and incidence of TB in India

1.3.1 TB/HIV Co-infection

The emergence and spread of HIV and drug-resistant tuberculosis further threaten to complicate the tuberculosis situation in the country. India, the third highest HIV burdened country, had an estimated 2.31 million (0.36% of adult population in the country) people living with HIV/AIDS (PLHAs) in 2006, emphasizing the enormous challenge ahead. All States and Union Territories of the country have reported HIV/AIDS cases. However, the HIV epidemic pattern shows great variance across the country. The worst affected states are Andhra Pradesh, Karnataka, Maharashtra, Manipur, Nagaland and Tamil Nadu. These six states have reported more than 75% of all the AIDS cases in India and are classified as High Prevalence States. Three other states namely Goa, Gujarat and Pondicherry, have been classified as Moderate HIV prevalence states. Even within the high prevalence states, there are districts which have ante-natal HIV levels below 1%.

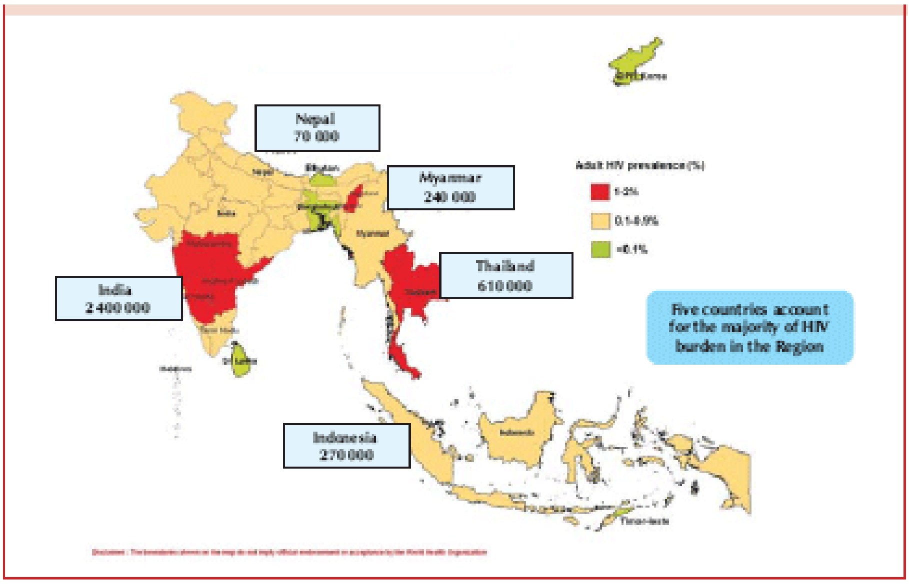


Figure 3: HIV prevalence in SEA region
‡ Source: Report on the Global AIDS Epidemic: UNAIDS, 2008

1.3.1.1 MDR and XDR-TB

"Drug resistant tuberculosis" is a man made problem. While tuberculosis is hundred percent curable, multidrug resistant tuberculosis (MDR-TB) is difficult to treat. Inadequate and incomplete treatment and poor treatment adherence has led to a newer form of drug resistance known as extensively drug resistant tuberculosis (XDR-TB). XDR-TB is defined as tuberculosis caused by Mycobacterium tuberculosis strain,

which is resistant to at least rifampicin and isoniazid among the first line anti tubercular drugs (MDR-TB) in addition to resistance to any fluroquinolones and at least one of three injectable second line anti tubercular drugs i.e. amikacin, kanamycin and/or capreomycin. Mismanagement of tuberculosis paves the way to drug resistant tuberculosis. The emergence of resistance to drugs used to treat TB, and particularly MDR-TB, has become a significant public health problem in a number of countries and an obstacle to effective TB control. Several small surveys conducted across the country have shown the prevalence rates of MDR-TB in the country at around 3% among new cases, and 12% among retreatment cases.

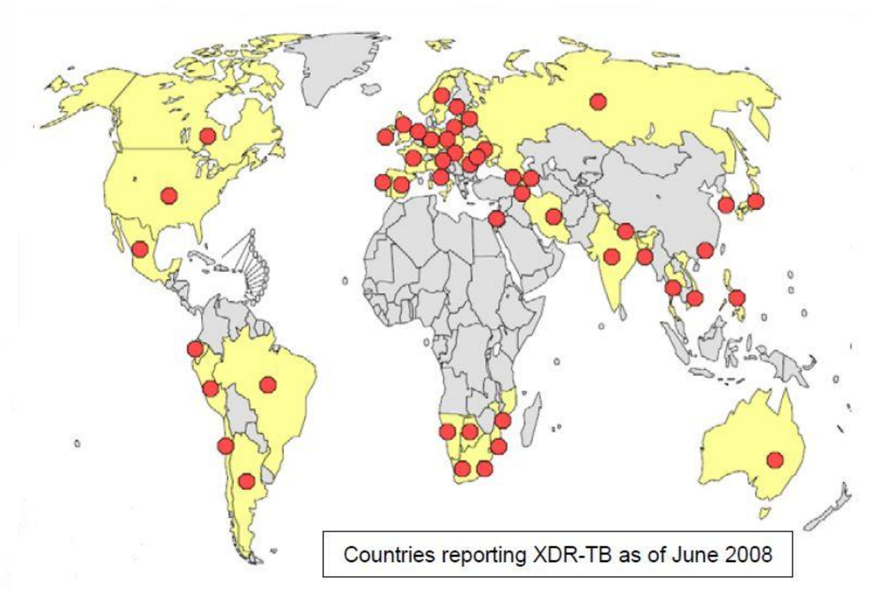


Figure 4: Countries reporting XDR-TB as on June 2008.
$\star$ *Source : www.who.int/tb/challenges/mdr/mdrtbfacts_june08.pdf*

A large scale population based survey in the states of Gujarat and Maharashtra has also indicated similar resistance levels (new-3% and retreatment- 12-17%). Available information suggests that the proportion of MDR-TB is relatively low in India. However, this translates into a large absolute number of cases, with an estimated annual incidence of 110,000 cases of MDR-TB. XDR-TB has been reported in India by isolated studies with non-representative and highly selected clinical samples. The magnitude of the problem remains to be determined due to the absence of laboratories capable of conducting quality assured second line Drug Susceptibility Testing (DST). However, what is frightening is the potential threat of XDR-TB in India, with unregulated availability and injudicious use of the second line drugs along with non-existence of systems to ensure standardized regimens and treatment adherence for MDR-TB outside the national programme. The problem of MDR and XDR-TB in India and across the world raises the possibility that the current TB epidemic of mostly drug susceptible TB will be replaced with a form of TB with severely restricted treatment options. If this happens it would jeopardize the progress made in recent

years to control TB globally as well as in India and would also put at risk the plans to progress towards a world where TB ceases to be a public health problem. India had a National Tuberculosis Programme (NTP) in place from the sixties, following epidemiological assessment of the situation during 1955-1958. There were serious limitations in the diagnostic paradigm and treatment regimens followed. India began its Revised National Tuberculosis Control Program (RNTCP) in 1993. Its mainstay is the strategy of directly observed treatment, short course (DOTS). Typically, during the initial 2 to 3 months of treatment, medication is administered three times a week under direct observation. During the subsequent 4 to 5 months, at least one of the three weekly administrations is directly supervised.

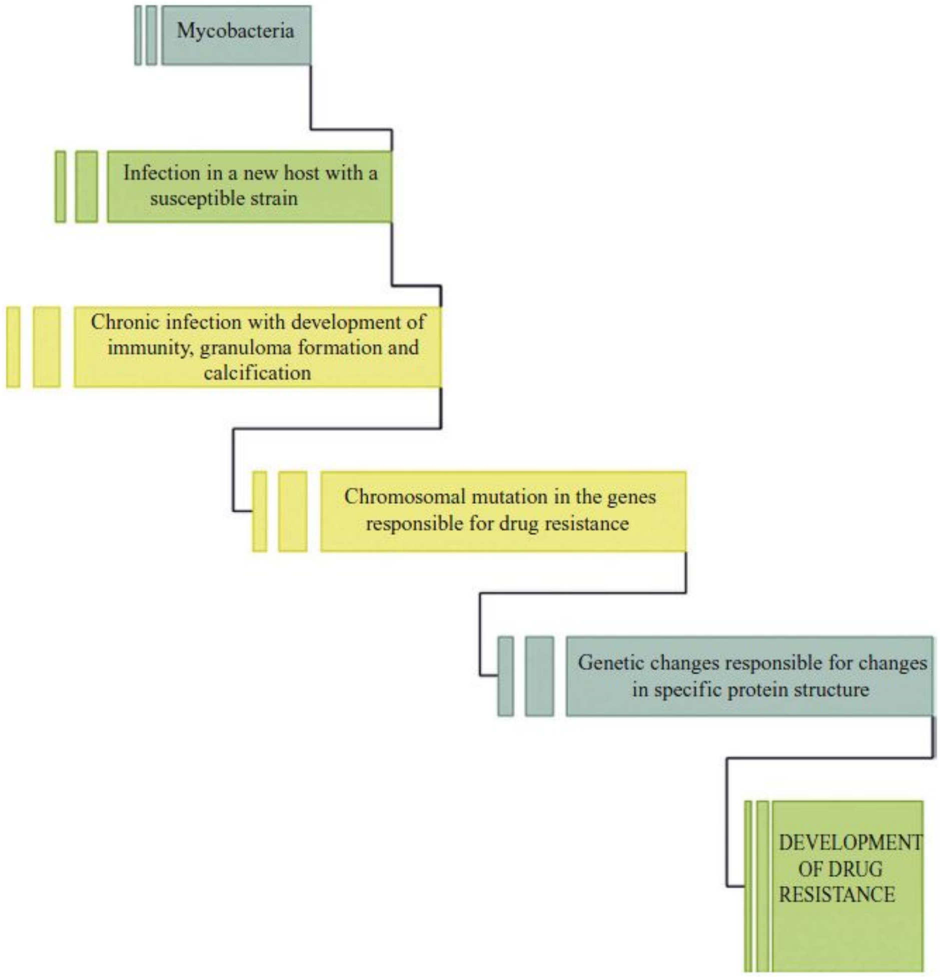


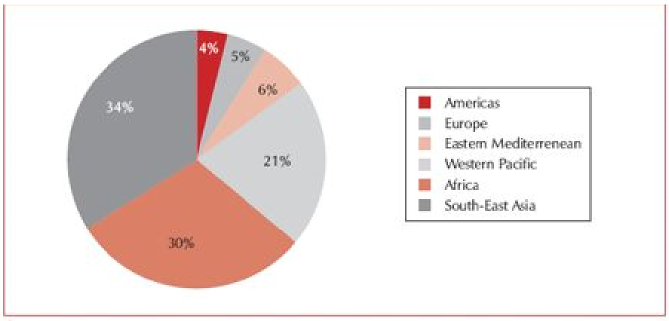
Figure 5: Mechanism of development of drug resistance in mycobacteria.
‡ *www.cdc.gov/tb.*

S.No.	Factors	Description	References
1.	Genetic factors	• Accumulation of changes in genomic content • Gene acquisition and loss • Spontaneous mutation	(Kato-Maeda *et al* 2001). (Sharma and Mohan 2004; Ramaswamy *et al* 1998)
2.	Incomplete and Inadequate treatment	• History of treatment of TB • Shortage of drugs • Increased cost of drugs • Physician error (drugs, dosing interval and duration) • Use of single drug to treat TB • Addition of a single drug to a failing regimen • Failure to identify preexisting resistance • Initiation of an inadequate primary regimen • Variations in bioavailability of anti-TB drugs	 (Mwinga 2001) (Chan and Iseman 2002) (Iseman 1993) (Sharma and Mohan 2003)
3.	Inadequate treatment adherence	• Poor compliance • Psychiatric illness • Alcoholism • Drug addiction • Homelessness • Travel to different places • Symptom relief • Adverse drug reactions • Inability to afford treatment • Length of treatment • Adverse drug reactions	(Jacaban 1994; Malian and Adam 1995; Goble *et al* 1993) (Sharma and Mohan 2004) (Johnson *et al* 2003)
4.	Other factors	• Poor infrastructure of NTCP • Poor administrative control on purchase and distribution of the drugs • No proper mechanism on quality control • No proper mechanism of bioavailability tests • No appropriate laboratory support leading to over diagnosis of TB • Unnecessary treatment • Side effects without benefit • Service inefficiencies • No specific therapy for MDR-TB DOTS strategy	(Prasad 2005) (Floyd *et al* 2006) (Sharma and Mohan 2006)

Table 4: Factors associated with development of MDR-TB.
† *www.cdc.gov/tb.*

1.4 Tuberculosis across globe

The South-East Asia Region, with an estimated 4.88 million prevalent cases and an annual incidence of 3.17 million TB cases, carries one-third of the global burden of TB (Figure 1). Five of the 11 member countries in the region are among the 22 high burden countries, with India accounting for over 20% of the world's cases. Most cases occur in the age group of 15-54 years, with males being disproportionately effected. The male/female ratio among newly detected cases is 2:1. Though deaths due to TB have declined after the introduction of DOTS. The control of tuberculosis



Source: Global Tuberculosis Control Surveillance, Planning, Financing, WHO, Geneva, 2009

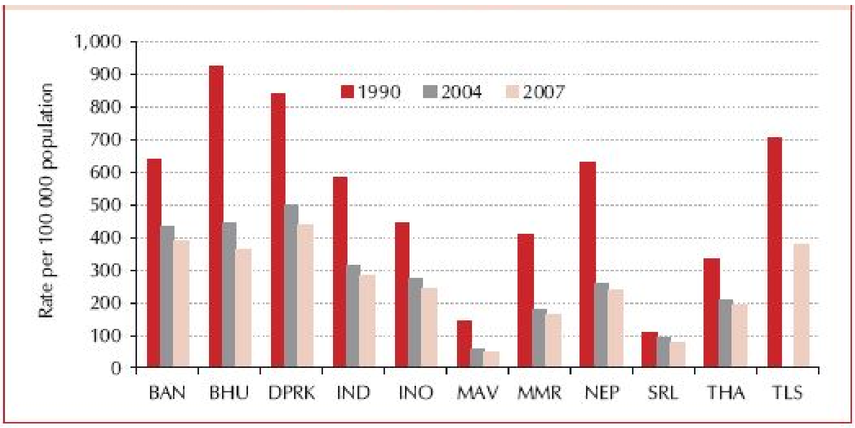
Figure 6: Estimated incidence of all forms of TB (WHO,2009).

in the region is affected by variations in the quality and coverage of various TB control interventions, population demographics, urbanization, changes in socio-economic standards, HIV and, more recently, emerging drug resistance.
However, An increasing morbidity and mortality from tuberculosis (TB) was predicted for the world at large, with the number of newly occurring cases predicted to increase from 7.5 million a year in 1990 to 8.8, 10.2 and 11.9 million in the years 1995, 2002 and 2005 respectively; an increase amounting to 58.6 per cent over a 15-yr period. The association with HIV and increasing multi drug resistant tuberculosis (MDRTB) appears to be a serious issue, especially for the developing nations.

Country	Population* (in 1000's)	Estimated Annual Incidence rate/100 000 population		Estimated Prevalence rate per 100 000 pop. – all forms of TB	Estimated death rate per 100 000 pop. – all forms of TB
		All cases	SS+ cases		
Bangladesh	158,665	223	100	387	45
Bhutan	658	246	110	363	44
DPR Korea	23 790	344	155	441	65
India	1 169 016	168	75	283	28
Indonesia	231 627	228	102	244	39
Maldives	306	47	21	48	4
Myanmar	48 798	171	75	162	13
Nepal	28 196	173	77	240	23
Sri Lanka	19 299	60	27	79	8
Thailand	63 884	142	62	192	21
Timor-Leste	1155	322	145	378	47
SEAR	**1 745 394**	**181**	**81**	**280**	**31**

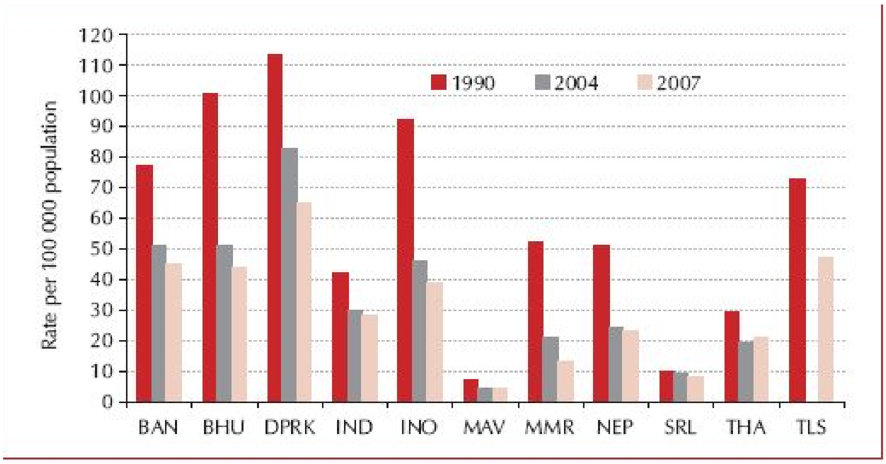
* UN Population Division, World Population Reports, 2007, New York (Rev.)

Table 5: The estimated TB incidence, prevalence and mortality rates for countries in the Region.



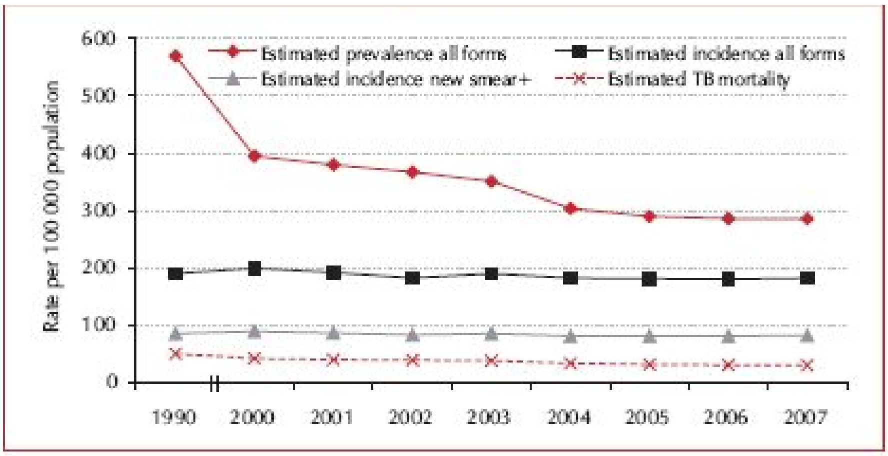
Source: Global Tuberculosis Control, WHO Reports 2001-2008

Figure 7: The estimated TB prevalence rates in the 11 Member countries of the Region comparing the rates between 1990, 2004 and 2007. These are indicative of a decrease in all countries of the region.



Source: Global Tuberculosis Control, WHO Reports 2001-2008

Figure 8: The estimated TB mortality rates for all forms of tuberculosis per 100 000 population, comparing the rates between 1990, 2004 and 2007. With respect to 1990, a significant decrease is observed in 2007 in all countries of the region.



Source: Global Tuberculosis Control: WHO Reports, 2001-2008

Figure 9: The overall trends in the estimated TB prevalence, incidence and mortality rates per 100 000 population in the Region as a whole, between 1990 and 2007. The estimated prevalence and mortality rates decreased slowly between 2004 and 2007.

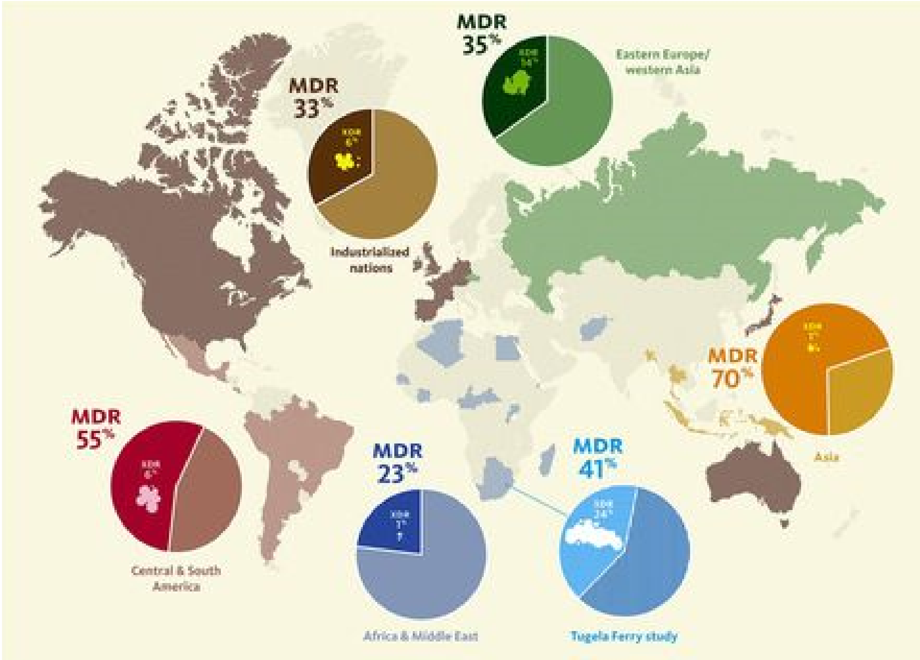


Figure 10: percentage of MDR found in different countries.
† *Source* : *www.yalealumnimagazine.com.*

1.5 Genome

Mycobacterium tuberculosis Genome is one of the largest (4.40 Mb) found till date, exceeded only by E. Coli (4.60 Mb genome) and Pseudomonas aeruginosa (6.26 Mb), and contains around 4,000 genes. Only about 40% of the genes have been given precise functions and 16% of its genes resemble no known proteins, most probably they are responsible for specific mycobacterial functions. More than 250 genes are devoted to lipid metabolism (E. coli has only about 50 such genes), and M. tuberculosis may obtain much of its energy by degrading host lipids. There are surprisingly large numbers of regulatory elements in the genome. This may mean that infection process is much more complex and sophisticated than previously thought. Two families of novel glycine-rich proteins with unknown functions are present and represent about 10% of the genome. They may be a source of antigenic variation and involved in defence against the host immune system. One of the major medical problems has been the lack of a good vaccine. A large number of proteins that are either secreted by the bacterium or on the bacterial surface have been identified from the genome sequence. It is hoped that some of these proteins can be used to develop newer, more effective vaccines. This is particularly important in view of the spread of multiply drug resistant M. tuberculosis.

It is tempting to think that closely related and superficially similar bacteria must have similar genomes. The genome of the leprosy bacillus, Mycobacterium leprae, shows that this assumption can be mistaken. The whole M. leprae genome is a third

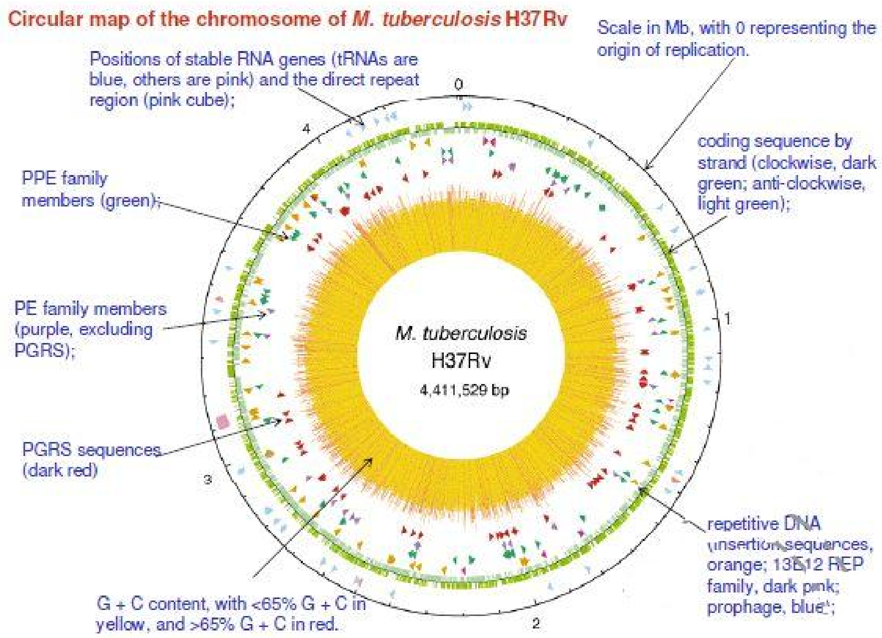


Figure 11: Genome components of Mycobacteria tuberculosis H37Rv.
$*$ *Source* : *www.iayork.com.*
† *Source* : *www.science.smith.edu/departments/.../Grob − Final − Tuberculosis.ppt*

smaller than that of M. tuberculosis. About half the genome seems to be devoid of functional genes, it consists of junk DNA that represents over 1,000 degraded, non-functional genes. In total, M. leprae seems to have lost as many as 2,000 genes during its career as an intracellular parasite. It even lacks some of the enzymes required for energy production and DNA replication. This might explain why the bacterium has such a long doubling time, about two weeks in mice. One hope from genomics studies is that critical surface proteins can be discovered and used to develop a sensitive test for early detection of leprosy. This would allow immediate treatment of the disease before nerve damage occurs.

1.6 Evolution

Earlier it was thought that tuberculosis has been present in humans since the ancient times and the source of the disease was said to be harbouring in cattle. But this has been disproved by an evolutionary concept which used analysis of sequences deleted from various species of Mycobacterium tuberculosis complex. A recent DNA-based study (Gibbons et al, 2008), has shown that humans were affected by TB long before they started cattle rearing. So, it may be believed that it was humans who transferred this disease to animals. The recent phylogenetic studies (Pfister et al., 2008) suggest that a proto M. tuberculosis split from the mycobacterium that causes leprosy about 36 million years ago, perhaps in a primate ancestor of humans. It also estimates that M. tuberculosis in humans gave rise to M. bovis, the strain that infects cattle,

about 113,000 years ago, and to the strains in several other mammals about 90,000 to 100,000 years ago. Since then, M. tuberculosis has undergone genome downsizing and extensive lateral gene transfer to become a specialized pathogen of humans and other primates without retaining an environmental niche (Stinear et al., 2008).

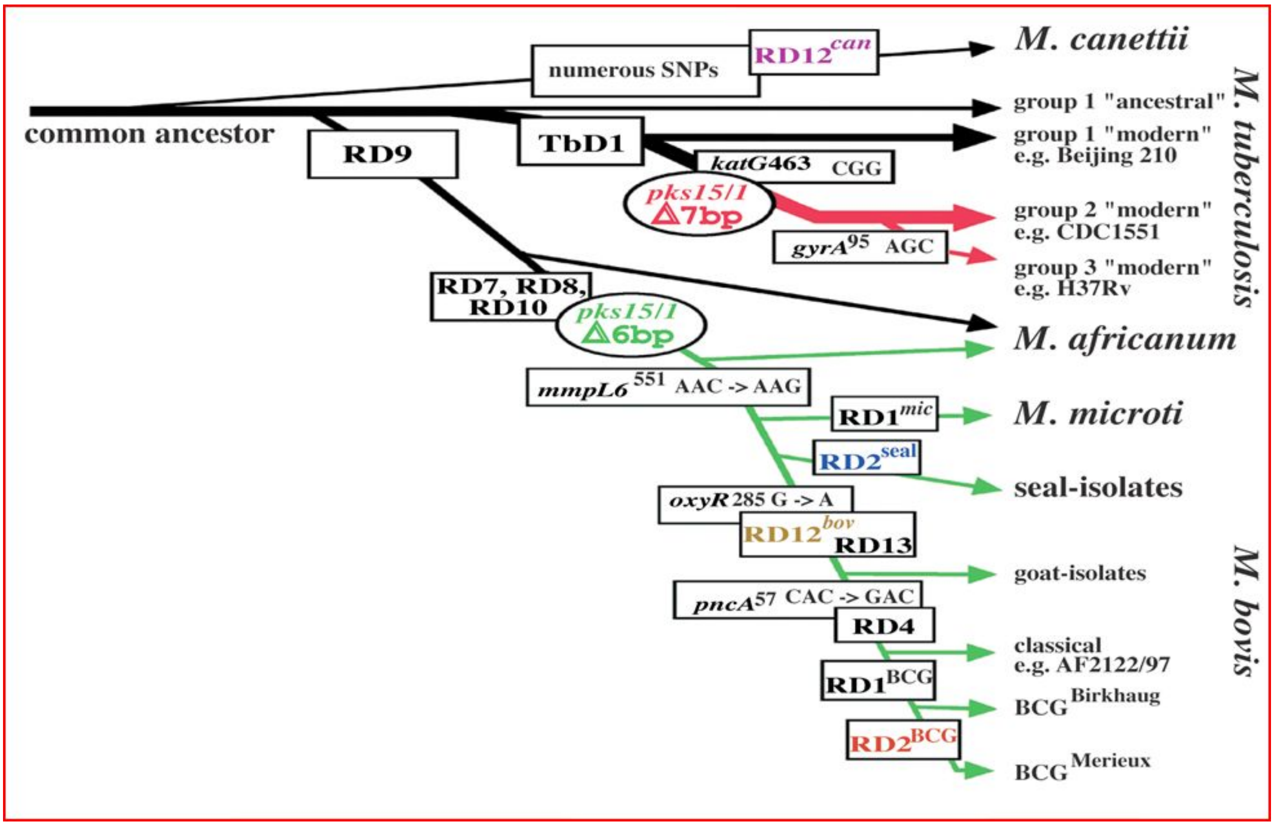


Figure 12: Scheme of Evolutionary pathway of tubercle bacilli illustrating successive loss of DNA in certain lineages. The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes where RD represents Region of Difference, genomic deletion. Distance between certain branches may not correspond to actual phylogenetic differences calculated by other methods (Hershberg et al).

$* Source : www3.ensp.fiocruz.br/eventos\_novo/dados/arq275.ppt$

Studies have also proved that Mycobacterium tuberculosis which was once considered genetically uniform, has imbibed zoogeographical variation as a consequence of human migrations (Hershberg et al). In the apparent absence of purifying selection, many of the mutations are retained and result in non-synonymous changes in amino acids, which are likely to have functional effects. It is not clear how M. tuberculosis tolerates the potentially deleterious consequences of genetic drift, but this cryptic variation needs to be taken into account in designing vaccines and drugs.

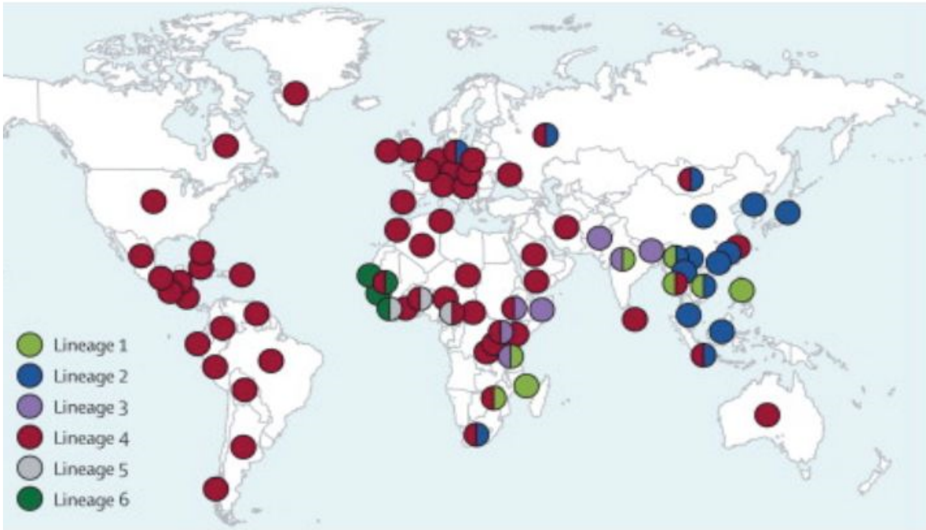


Figure 13: The six main lineages as a result of migration (Reed et al).
∗ *Source* : *www.nimr.mrc.ac.uk*

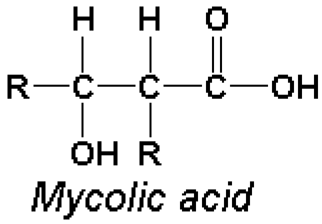
There were found to be six main lineages of M. tuberculosis that were geographically structured. Recently, it was discovered that the Beijing family of Mycobacterium tuberculosis is the most disastrous as it has a new glycolipid of hyper virulent tuberculosis strains that inhibits the innate immune response (Reed et al). This new lipid PGL increases lethality greatly but not bacterial load; down regulates pro-inflammatory response in dose-dependent manner, represses TNF α, IL-6 and IL-12 production and may contribute to increased transmission along with MDR.

1.7 Morphology of Mycobacteria

Mycobacteria are typically slender, straight or slightly curved rods (average 3.0 x 0.3μm). They are aerobic, non motile, non capsulated, non sporing and sometimes show branching filamentous forms resembling fungal mycelia occurring singly, in pairs or as small clumps. The size depends on conditions of growth, and long filamentous, club shaped and branching forms may be sometimes seen.

Tuberculi bacilli have been described as gram positive, though strictly speaking this is not correct, as after staining with basic dyes they resist decolourisation by alcohol even without the nordanting effect of iodine. When stained with carbol fuchsin by the Ziehl-Neelsen method or by fluorescent dyes, they resist decolourisation by 20% sulfuric acid and absolute alcohol for 10 minutes (acid and alcohol fast).

Acid fastness has been ascribed variously to the presence in the bacillus of an unsaponifiable wax (mycolic acid) or to a semi permeable membrane around the cell. It is related to the integrity of the cell and appears to be a property of the lipid rich waxy cell wall.



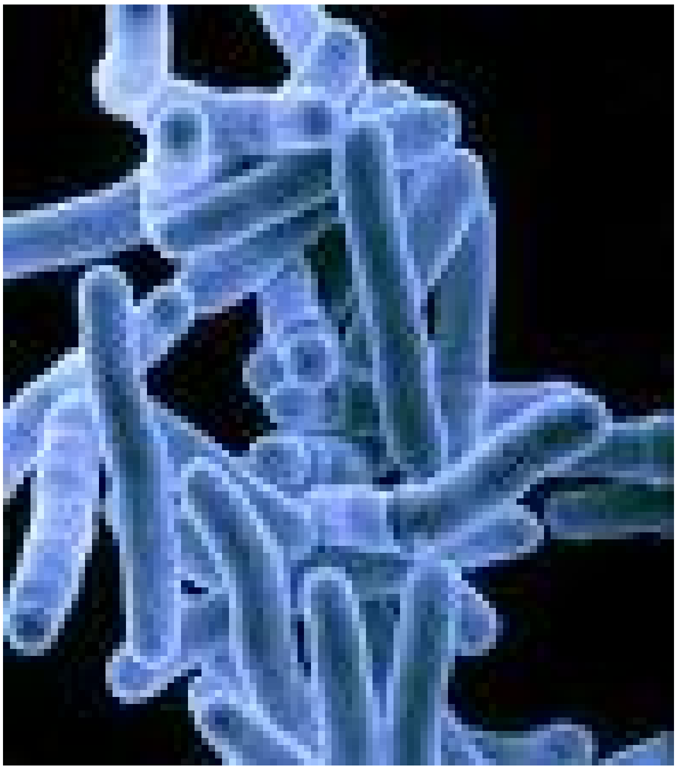


Figure 14: Electron micrograph picture of M. tuberculosis.
‡ *Source* : *www.nature.com*

Two media are used to grow M. TB. Middlebrook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium. M.TB. colonies are small and buff coloured when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing M.TB. It takes 4-6 weeks to get visual colonies on either type of media. Chains of cells in smears made from in-vitro-grown colonies often form distinctive serpentine cords. This observation was first made by Robert Koch who associated cord factor with virulent strains of the bacterium. MTB can be classified as gram positive due to their lack of an outer cell membrane.

1.7.1 Cultural characteristics

The bacilli grow slowly, the generation time in-vitro being 14-15 hours. Colonies appear in about 2 weeks and may sometimes take upto 8 weeks. Optimum temperature is 37°C and growth does not occur below 25°C or above with optimum pH of 6.4-7.0. M. tuberculosis is an obligate aerobe, while M. bovis is microaerophilic on primary isolation, becoming aerobic on subculture. M. tuberculosis grows luxuriantly in culture as compared to M. bovis which grows sparsely. They are therefore termed "eugonic and dysgonic" respectively. The addition of 0.5% glycerol improves the growth of MTB. Tubercle bacilli do not have exacting growth requirements but are highly susceptible even to traces of toxic substances like fatty acids in culture media. The toxicity is neutralized by serum albumin. Several media, both solid and liquid have been described for the cultivation of tubercle bacilli. The solid media contain egg (Lowenstein Jensen, Petragnini, Dorset). Blood (Tarshis), serum (Loeffler).

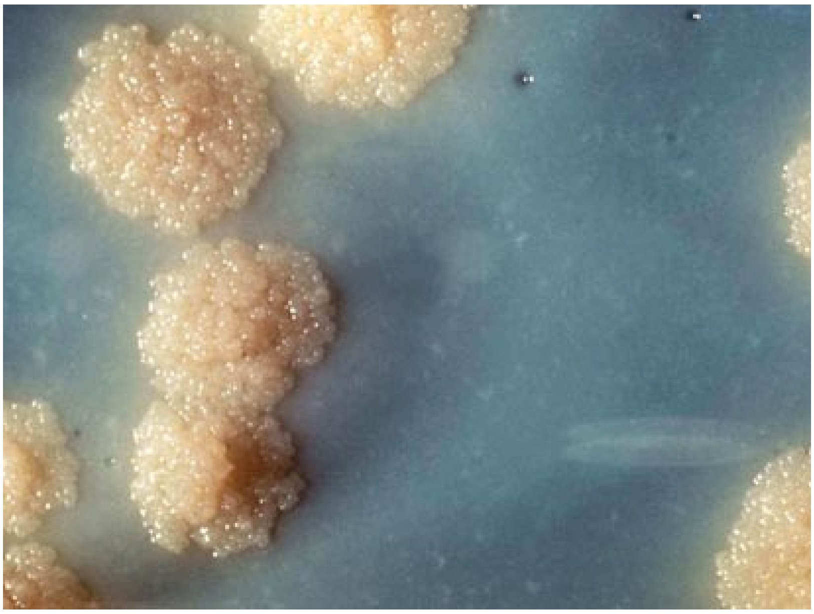


Figure 15: Typical small, buff coloured colonies of tuberculosis on Lowenstein Jensen medium.

‡ *Source* : *ilovebacteria.com*

The solid medium most widely employed for routine culture is Lowenstein-Jensen (LJ) medium without starch, as recommended by the International Union Against Tuberculosis (IUAT). This consists of coagulated hen's egg, mineral salt solution, asparagines and malachite green, the last acting as a selective agent inhibiting other bacteria. Among liquid media, Middlebrook media is more common. On solid media MTB form a dry, rough, raised, irregular colonies with a wrinkled surface. They are creamy white, becoming yellowish or buff coloured on further incubation. They are

tenacious and not easily emulsified. M. bovis colonies in comparison are flat, smooth, moist, white and break up easily when touched. In liquid media without dispersing agents the growth begins at the bottom, creeps up the sides and forma a prominent surface pellicle which may extend along the sides above the medium. Virulent strains tend to form long serpentine cords in liquid media, while avirulent strains grow in a dispersed manner.

1.7.1.1 Resistance

Mycobacteria are not specially heat resistant, being killed at 60°C in 15-20 minutes. Survival is influenced by the material in which bacteria are present. Cultures may be killed by exposure to direct sunlight for two hours, but bacilli in sputum may remain alive for 20-30 hours. Bacilli in droplet nuclei may retain viability for 8-10 days under suitable conditions. Cultures remain viable at room temperature for 6-8 months and may be stored up to two years at -20°C. Tubercle bacilli are relatively resistant to chemical disinfectants, surviving exposure to 5% phenol, 15% sulphuric acid, 3% nitric acid, 5% oxalic acid and 4% sodium hydroxide. They are sensitive to formaldehyde and gluteraldehyde. They are destroyed by tincture of iodine in five minutes and by 80% ethanol in 2-10 minutes. Ethanol is a suitable disinfectant for skin, gloves and clinical thermometer.

1.8 Biochemical reactions

Several biochemical tests have been described for the identification of mycobacterial species.

Niacin test: Human tubercle bacilli form niacin when grown on an egg medium. When 10% cyanogen bromide and 4% aniline in 96% ethanol are added to a suspension of the culture, a canary yellow colour indicates a positive reaction. The test is positive with human type and negative with bovine type of bacilli. The test is useful in identifying human strains as no other mycobacterium is positive, except for M. simiae and a few strains of M. cheloneii.

Aryl sulphatase test: The test is positive only with atypical mycobacteria. The bacilli are grown in a medium containing 0.001 M tripotassium phenolphthalein disulphate. 2 N NaOH is added drop by drop to the culture. A pink colour indicates a positive reaction.

Neutral red test: Virulent strains of tubercle bacilli are able to bind neutral red in alkaline buffer solutions, while a virulent strains are unable to do so.

Catalase-Peroxidase tests: These help in differentiating tubercle bacilli from atypical mycobacteria and provide an indication of the sensitivity of the strain to isoniazid. Most atypical mycobacterial strains are strongly catalase positive, while tubercle bacilli are only weakly positive in comparison. On the other hand, tubercle bacilli are peroxidase positive, but not atypical mycobacteria. Catalse and peroxidase activities are lost when tubercle bacilli become INH resistant.

Catalase negative strains of tubercle bacilli are not virulent for guinea pigs. A mixture of equal volumes of 30 vol. Hydrogen peroxide and 0.2% catechol in distilled water is added to 5 ml of the test culture and allowed to stand for few minutes. Effervescence indicates catalase production and browning indicates peroxidase activity.

Amidase tests: The ability to split amides has been used to differentiate mycobacteria. A useful pattern is provided by testing five amides, via, acetamide, benzamide, carbamide, nicotinamide and pyrazinamide. A 0/00165 M solution of the amide is incubated with the bacillary suspension at 37 degrees and 0.1 ml of hypochlorite solution are added. The tubes are placed in boiling water for 20 minutes. A blue color indicates a positive test.

Figure 16: Sputum smear showing Mycobacteria.
$* \, Source : http : //www.answers.com/topic/tuberculosis$

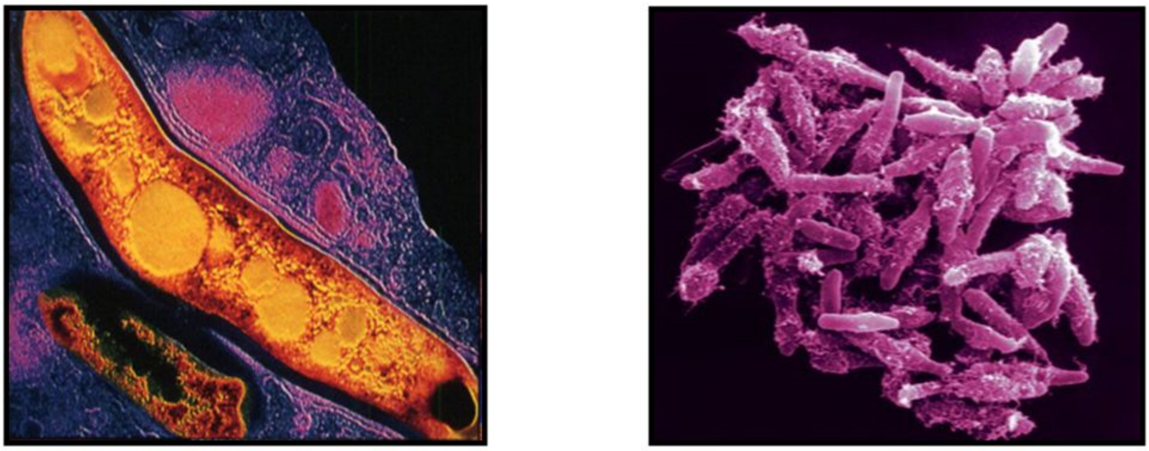


Figure 17: Mycobacterium tuberculosis.
‡ *Source* : *www.topnews.in/healthcare/diseases/tuberculosis*

1.8.1 Antigenic properties

Many antigens have been identified in mycobacteria. Group specificity is due to polysaccharide and type specificity to protein antigens. Following infection by tubercle bacilli, delayed hypersensitivity is developed to the bacillary protein (tuberculin). Tuberculin's from human, bovine and murine bacilli appear to be indistinguishable. Some degree of antigenic relationship exists between tubercle bacilli and atypical mycobacteria, as shown by weak cross reactions in skin testing with different tuberculin's. There is also some antigenic relationship between lepra and tubercle bacilli. By various serological tests it has been established that M. tuberculosis strains are antigenically homogenous and very similar to M. bovis and M. microti, but distinct from other species. Antibodies against polysaccharide, protein and phosphate antigens of tubercle bacilli have been demonstrated in sera of patients, but they have not been found useful in diagnosis or relevant in immunity.

1.8.2 Cell wall

The typical cell morphology of Mycobacterium tuberculosis as seen in acid fast stain, as a thin slightly curved bacillus measuring 0.3 to 0.6 by 1 to 4 μm, deeply red staining (strongly acid fast), with a distinct beaded appearance. Stains differ in tendency to grow as discrete rods or as aggregated long strands called serpentine rods. Optimal growth is seen at 37°C. They do not grow below 25°C, and optimum pH ranges between 6.4 and 7.0 but growth is best at pH 6.8. The higher susceptibility of M. tuberculosis to acid pH is due to its poor ability to maintain pH homeostasis.

The cell wall structure of Mycobacterium tuberculosis deserves special attention because it is unique among prokaryotes and it is a major determinant of virulence for the bacterium. The Mycobacterium tuberculosis cell envelope differs substantially from the cell wall structures of both Gram-negative and Gram-positive bacteria. The

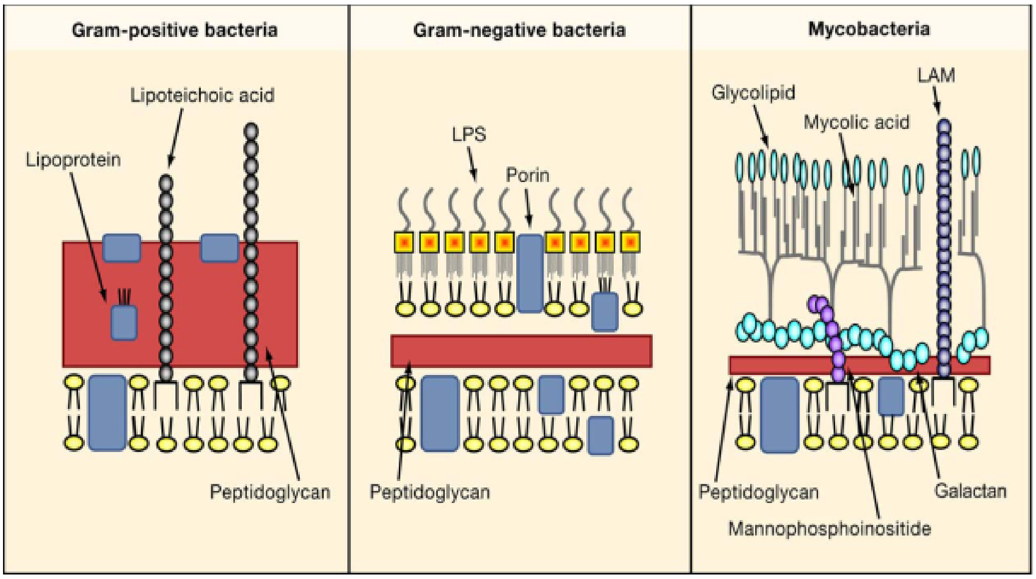


Figure 18: Schematic Representation of Bacterial Cell Walls Gram-positive bacteria have a thick layer of PG. Lipoteichoic acids and lipoproteins are embedded in this cell wall. The cell wall of Gram-negative bacteria is characterized by the presence of LPS. Mycobacteria have a thick hydrophobic layer containing mycolyl arabinogalactan and dimycolate, in addition to a lipid bilayer and a PG layer. Lipoarabinomannan (LAM) is a major cell-wall-associated glycolipid. Lipoproteins are common structures for various types of bacteria.

$* \, Source : Review \ by \ Shizuo \ Akira, 2006.$

main structural element consists of a cross-linked network of peptidoglycan (PG) in which some of the muramic acid residues are replaced.

With a complex polysaccharide, Arabinogalactan (AG), AG is attached to PG through a unique linker unit, and in turn is acylated at its distal end to PG with mycolic acids. The entire complex is abbreviated as the mycolylarabinogalactan-peptidoglycan (mAGP) and is essential for viability in M. tuberculosis and other mycobacteria.

Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of M. tuberculosis's cell wall consists of Mycolic acids, which are unique α-branched lipids found in cell walls of Mycobacterium and Corynebacterium. They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic Acids are thought to be a significant determinant of virulence in M. TB. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum. Cord factor (trehalose dimycolate) in the bacterial walls can disrupt the respiration of mitochondria in phagocytes and tissue cells. Strains of M. tuberculosis possessing cord factor are virulent; strains lacking it are not. In cultures, the presence of cord factor is indicated when rough-looking colonies are formed due to growth of

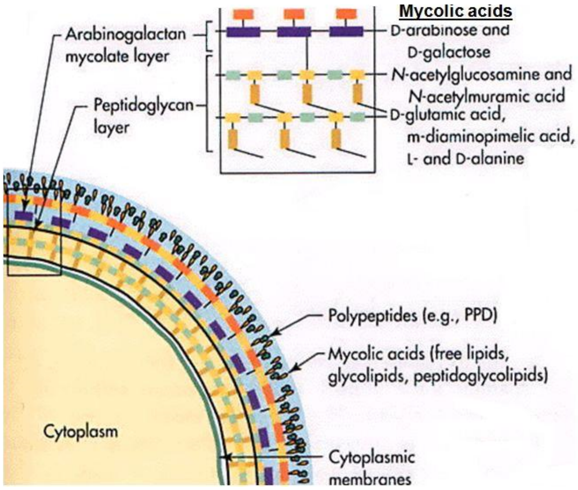


Figure 19: Components of cell wall.

the bacteria in cable like arrangements (cords).

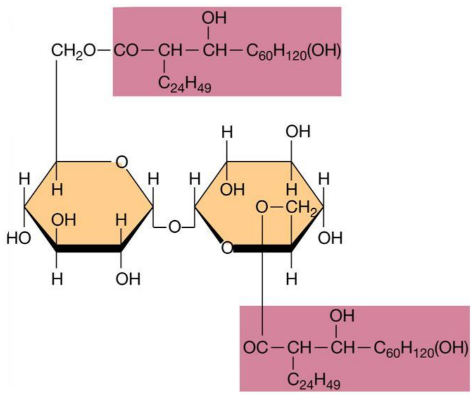


Figure 20: Structure of cord factor, a mycobacterial glycolipid 6, 6 dimycolyltrehalose.

‡ *Source* : *www.chem.usu.edu*

In summary, the high concentration of lipids in the cell wall of M. tuberculosis has been associated with these properties of the bacterium :-

- Impermeability to stains and dyes
- Resistance to many antibiotics
- Resistance to killing by acidic and alkaline compounds
- Resistance to osmotic lysis via complement deposition
- Resistance to lethal oxidations and survival inside of macrophages (Hussain, 2007).

1.9 Natural history of Tuberculosis

The mode of infection is by direct inhalation of aerosolize bacilli contained in droplet nuclei of expectorated sputum, sneezing, Coughing and speaking release numerous droplets - as many as 3000 infectious nuclei per cough. Dried bacilli in dust are much less infectious.

Figure 21: Sharma and Mohran (2004) reviewed the transmission of tuberculosis. M. tuberculosis is transmitted via water droplets that are expelled when a patient with active TB coughs, sneezes, or talks. Active TB patients are those with lesions in their lungs due to bacterial invasion. They reviewed that one droplet contains about three bacilli; talking for five minutes releases roughly 3000 droplets. The probability of infection with M. tuberculosis depends of the quantity expelled (infectiousness of the person with TB), the environment into which it is expelled, the duration of exposure, and the virulence of the organism (Reviewed by Sharma and Mohran, 2004). Once one of these droplets is inhaled and the droplet reaches alveoli in lungs, TB infection can begin.
† *Source* : *www.science.smith.edu/departments/.../Grob − Final − Tuberculosis.ppt*

The source of infection is usually an open case of pulmonary tuberculosis. It is estimated that an open case of tuberculosis in India may infect on an average some 25 contacts before death or cure. Other forms of tuberculosis are of much less importance in public health. Spread occurs most often among household or other close and prolonged contacts of open cases, whose sputum may have over 10,000 bacilli per ml. Infection also occurs infrequently by ingestion, for example, through infected milk and rarely by inoculation.

When the inhaled tuberculosis bacteria enter the lungs, they can multiply and cause a local lung infection (pneumonia). The local lymph nodes associated with the lungs

may also become involved with the infection and usually become enlarged. The hilar lymph nodes (the lymph nodes adjacent to the heart in the central part of the chest) are often involved. Tuberculosis enters into the lungs of its host (pulmonary T.B), where it slowly but regularly divides without causing any outward symptoms, but divides to attack its host. Macrophages that normally ingest pathogens in order to destroy them are invaded by the tubercle bacillus.

In addition, TB can spread to other parts of the body. The body's immune (defense) system, however, can fight off the infection and stop the bacteria from spreading. The immune system does so ultimately by forming scar tissue around the TB bacteria and isolating it from the rest of the body. Tuberculosis that occurs after initial exposure to the bacteria is often referred to as primary TB. If the body is able to form scar tissue (fibrosis) around the TB bacteria, then the infection is contained in an inactive state. Such an individual typically has no symptoms and cannot spread TB to other people. The scar tissue and lymph nodes may eventually harden, like stone, due to the process of calcification of the scars (deposition of calcium from the bloodstream in the scar tissue). These scars often appear on X-rays and imaging studies like round marbles and are referred to as a granuloma.

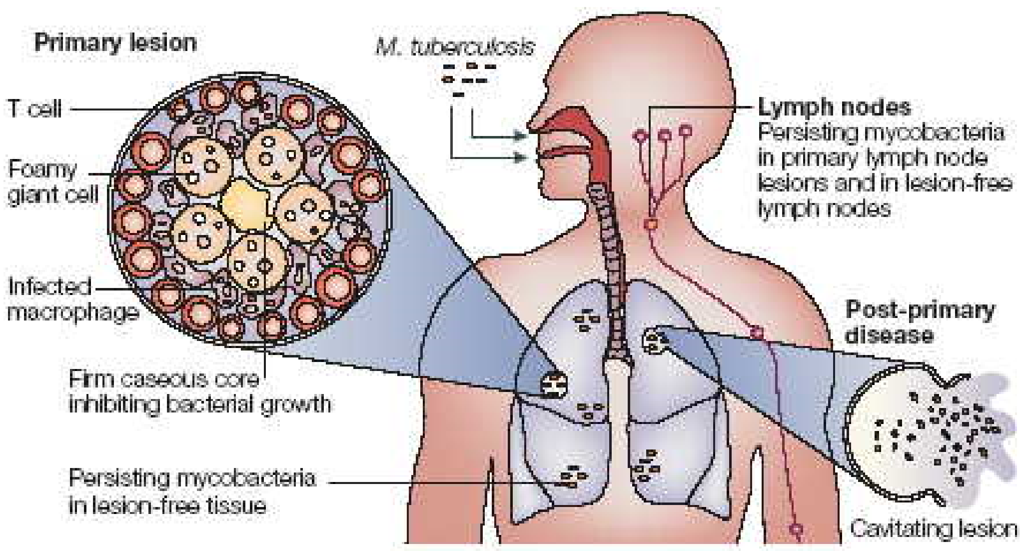


Figure 22: Pathogenesis of Mycobacterium Tuberculosis.
$\star$ *Source* : *pathport.vbi.vt.edu*

Sometimes, however, the body's immune system becomes weakened, and the TB bacteria break through the scar tissue and can cause active disease, referred to as reactivation tuberculosis or secondary TB. For example, the immune system can be weakened by old age, the development of another infection or a cancer, or certain medications such as cortisone, anticancer drugs, or certain medications used to treat arthritis or inflammatory bowel disease. The invasion of bacteria can result in a recurrence of the pneumonia and a spread of TB to other locations in the body.

The kidneys, bone, and lining of the brain and spinal cord (meninges) are the most common sites affected by the spread of TB beyond the lungs.

The essential pathology in tuberculosis is the production in infected tissues of a characteristic lesion, the tubercle. This is an avascular granuloma composed of a central zone containing giant cells with or without caseation and a peripheral zone of lymphocytes and fibroblasts.

Tuberculous lesions are primarily of two types - exudative and productive. The exudative type is an acute inflammatory reaction with accumulation of edema fluids, polymorphonuclear leucocytes and later of lymphocytes and mononuclear cells. This is typically seen when the bacilli are many and virulent and the host response is more in the nature of DTH than of protective immunity.

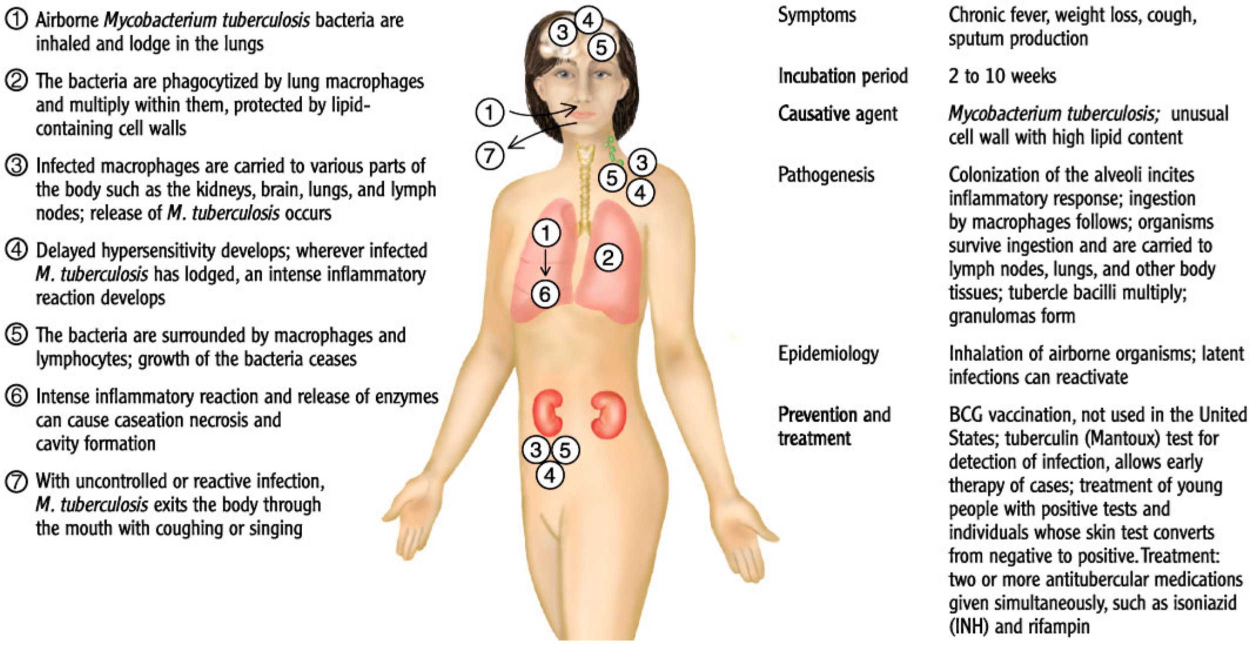


Figure 23: Events leading to the development of Tuberculosis.
⋆ *Source* : *Fundamentals in Microbiology*, *Tata McGraw Hill*

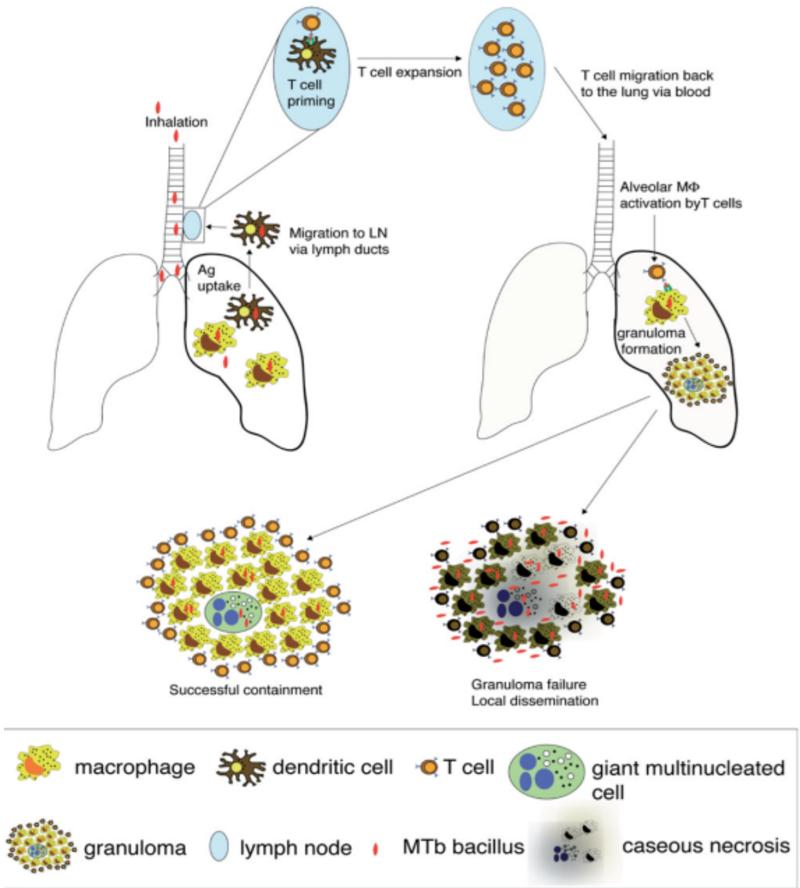


Figure 24: Events that follow infection with M. Tuberculosis.
⋆ *Source* : *www.nature.com.*

Types of disease :-
Tuberculosis is either latent or active.
Latent TB means that the TB bacteria exists in the body, but the body's defenses (immune system) fight the infection and try to keep it from turning into active TB. This means the absence of any symptoms of TB in initial stages and inability to spread the disease to others. However, the latent TB can become active TB.
Active TB means that the TB bacteria are growing and causing symptoms. If lungs are infected with active TB, it is easy to spread the disease to others.

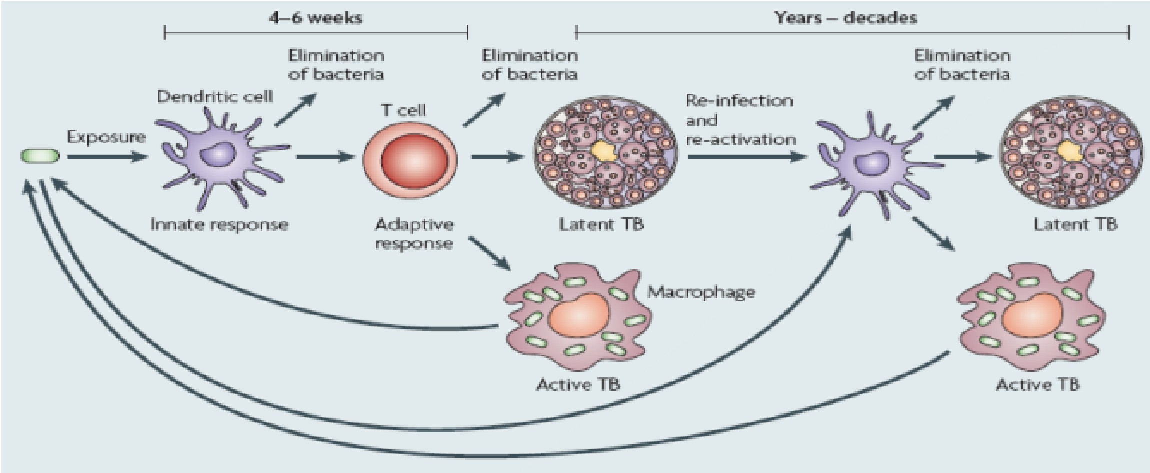


Figure 25: Steps involve from exposure to reinfection with time.
$*$ *Source* : *www.nature.com/.../n7/images/nrmicro*1919 – *i2.jpg*

1.10 Types of Tuberculosis

On the basis of clinical manifestations and site affected, tuberculosis can be divided into :-

1. Pulmonary tuberculosis (PTB)
2. Extra-pulmonary tuberculosis (EPTB)

Pulmonary tuberculosis (TB) is a contagious bacterial infection that mainly involves the lungs, but may spread to other organs. The primary stage of the infection is usually asymptomatic (without symptoms). However, in some cases, the disease may become active within weeks after the primary infection, or it may lie dormant for years and later reappear. Tuberculosis infection occurs as a consequence of the inhalation of bacillus-laden droplets expelled from an infected host. Once the organism is inhaled, it travels via the airways to the pulmonary parenchyma, where it is deposited. Although the organism may be deposited in any lobe, a predilection for the lower lobes exists. TB infection begins when the mycobacterium reach the pulmonary alveoli, where they invade and replicate within alveolar macrophages. The primary site of infection in the lungs is called the Ghon focus. Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymphnodes. Pulmonary Tuberculosis is further divided into 5 different types of TB. These include : -

1. Primary TB pneumonia: It is an uncommon form of TB, which mostly occurs in patients with lower immunity, like children and the elderly. It presents itself in the form of pneumonia and is highly contagious.
2. Laryngeal TB: It affects the throat, in the vocal chord area. It is also contagious.

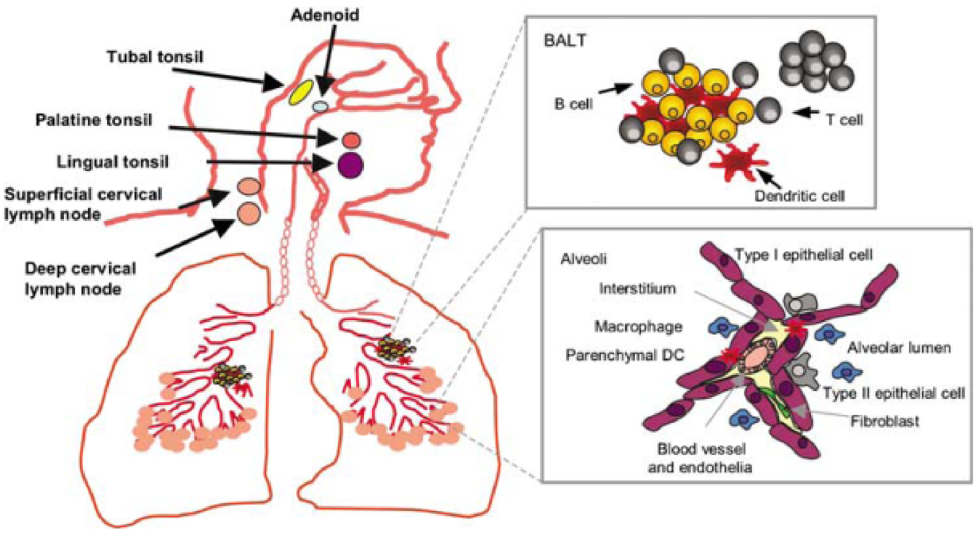


Figure 26: Mode of infection from mouth to lungs.
‡ *Source* : *www.nature.com*

3. Cavitary TB: Cavitary TB involves the upper lobes of the lung. The bacteria cause progressive lung destruction by forming cavities, or enlarged air spaces. This type of TB occurs in reactivation disease. The upper lobes of the lung are affected because they are highly oxygenated (an environment in which M. tuberculosis thrives). Cavitary TB can, rarely, occur soon after primary infection. Symptoms include productive cough, night sweats, fever, weight loss, and weakness. There may be hemoptysis (coughing up blood). Patients with cavitary TB are highly contagious. Occasionally, disease spreads into the pleural space and causes TB emphysema (pus in the pleural fluid).

4. Miliary TB: Miliary TB is disseminated TB. 'Miliary' describes the appearance on chest x-ray of very small nodules throughout the lungs that look like millet seeds. Miliary TB can occur shortly after primary infection. The patient becomes acutely ill with high fever and is in danger of dying. The disease also may lead to chronic illness and slow decline. Symptoms may include fever, night sweats, and weight loss. It can be difficult to diagnose because the initial chest x-ray may be normal. Patients who are immunosuppressed and children who have been exposed to the bacteria are at high risk for developing miliary TB.

5. Tuberculosis pleurisy: This usually develops soon after initial infection. A granuloma located at the edge of the lung ruptures into the pleural space, the space between the lungs and the chest wall. Usually, a couple of tablespoons of fluid can be found in the pleural space. Once the bacteria invade the space, the amount of fluid increases dramatically and compress the lung, causing shortness of breath (dyspnea) and sharp chest pain that worsens with a deep breath (pleurisy). A chest x-ray shows significant amounts of fluid. Mild- or low-grade fever commonly is present. Tuberculosis pleurisy generally resolves without

treatment; however, two-thirds of patients with tuberculosis pleurisy develop active pulmonary TB within 5 years.

Extra-pulmonary tuberculosis (EPTB) refers to disease outside the lungs. It is sometimes confused with non-respiratory disease. Disease of the larynx for example, which is part of the respiratory system, is respiratory but extra-pulmonary. Extra pulmonary TB is further divided into 7 different types of TB. They include the following :

1. Adrenal Tuberculosis: This form affects the adrenal gland; hence the hormone production is also affected. Patients suffering from this form of TB are known to experience fainting or weakness.

2. Lymph node disease: It is characterized by the patients experience enlargement of the lymph nodes. The nodes could also rupture through the skin.

3. Osteal Tuberculosis: This form of TB affects the bones. The affected area's bone tissue weakens, and it could cause the patient to fracture the affected area.

4. TB Peritonitis: It usually affects the outer lining of the intestine. Due to the TB, fluid gets collected in the outer lining of the intestine, causing the affected to experience pain in the abdomen.

5. Renal TB: It is characterized by the patient experiencing pyuria, which is the presence of white blood cells in the urine. It could end up affecting the reproductive organs and cause Epididymitis in men.

6. TB Meningitis: The symptoms for this include the patients displaying signs of being affected by a stroke or a brain tumor. It is extremely dangerous and could even prove to be fatal.

7. Tuberculosis pericarditis: The membrane surrounding the heart (the pericardium) is affected in this condition. This causes the space between the pericardium and the heart to fill with fluid, impeding the heart's ability to fill with blood and beat efficiently.

1.10.1 Symptoms of Tuberculosis

As previously mentioned, TB infection usually occurs initially in the upper part (lobe) of the lungs. The body's immune system, however, can stop the bacteria from continuing to reproduce. Thus, the immune system can make the lung infection inactive (dormant). On the other hand, if the body's immune system cannot contain the TB bacteria, the bacteria will reproduce (become active or reactivate) in the lungs and spread elsewhere in the body. It may take many months from the time the infection

initially gets into the lungs until symptoms develop. The usual symptoms that occur with an active TB infection are a generalized tiredness or weakness, weight loss,

fever, and night sweats. If the infection in the lung worsens, then further symptoms can include coughing, chest pain, coughing up of sputum (material from the lungs) and/or blood, and shortness of breath. If the infection spreads beyond the lungs, the symptoms will depend upon the organs involved.

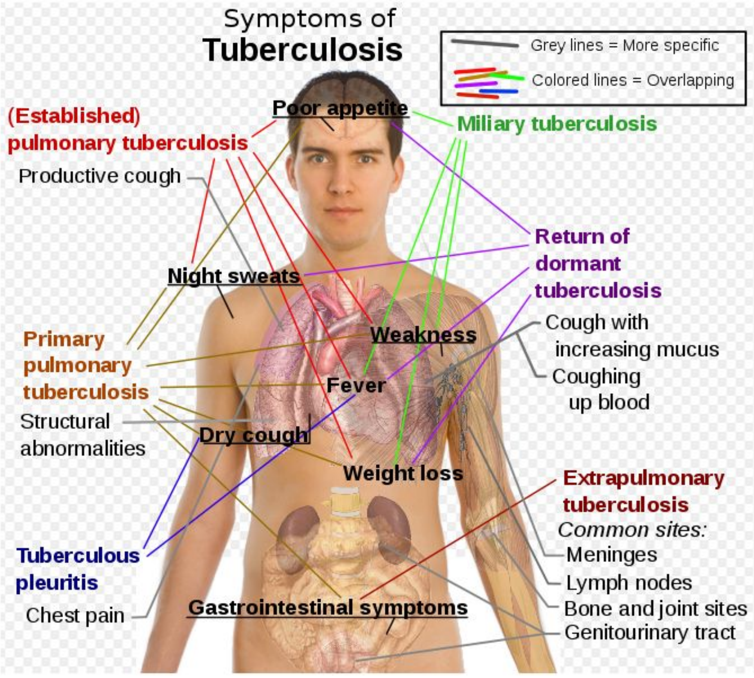


Figure 27: Symptoms of tuberculosis
† *Source : wikipedia.org/wiki/File : Tuberculosis$_s$ymptoms.svg*

1.10.2 Diagnosis of disease

TB can be diagnosed in several different ways, including chest X-rays, analysis of sputum, and skin tests. Sometimes, the chest X-rays can reveal evidence of active tuberculosis pneumonia. Other times, the X-rays may show scarring (fibrosis) or hardening (calcification) in the lungs, suggesting that the TB is contained and inactive. Examination of the sputum on a slide (smear) under the microscope can show the presence of the tuberculosis-like bacteria. Bacteria of the mycobacterium family, including atypical mycobacteria, stain positive with special dyes and are referred to as acid-fast bacteria (AFB). A sample of the sputum also is usually taken and grown (cultured) in special incubators so that the tuberculosis bacteria can subsequently be identified as tuberculosis or atypical tuberculosis. Several types of skin tests are used to screen for TB infection.

1.10.2.1 Delayed hypersensitivity reactions

When certain antigens are injected into the skin of a sensitized animal, an inflammatory response taking many hours to develop, may occur at the injection site. Since

this delayed hypersensitivity reaction cannot be transferred from sensitized to normal animals by serum, but only by the adoptive transfer of live lymphocytes, it is apparently cell mediated. The tuberculin response is an example of this type of hypersensitivity.

Tuberculin is the name given to extracts of M. tuberculosis (or closely related M. bovis or M. avium), which are employed as agents in the skin testing of humans or animals to identify those with tuberculosis. The most important extract being purified-protein-derivative (PPD) tuberculin. Its major antigenic component is probably HSP 65.

When PPD tuberculin is injected into the skin of a normal individual, there is no significant inflammation. On the other hand, if it is injected into a person sensitized by infection with the tubercle bacillus, a delayed hypersensitivity response occurs. Following injection of tuberculin into the skin of such an individual, very few changes are detectable for several hours. By 24 hours, however, local vasodilatation and increased vascular permeability occurs, which result in redness and the development of firm lump at the injection site. On histological examination, the reaction differs from a conventional acute inflammatory response in that the infiltrating cells are mononuclear cells, that is, macrophages and lymphocytes. A few neutrophils are found in the early stages of a reaction. The inflammation reaches its greatest intensity by 24 to 72 hours and may persist for several weeks before gradually fading. In very severe reactions, tissue destruction may occur at the injection site.

The tuberculin reaction is an immunologically specific inflammatory reaction mediated by T cells. When an individual develops tuberculosis, mycobacterial antigen is taken up by the antigen presenting cells and presented to CD4+ T cells. Some of these T cells respond by developing into a functional subgroup of T cells that can be called Tdh cells (dh-delayed hypersensitivity). Not all CD4+ T cells become dh cells. Indeed, the T- cell types involved are clearly heterogeneous; some are MHC class II+, and others are MHC II-. Once generated, Tdh cells can enter the circulation and respond to antigen entering the body by any route. Since delayed hypersensitivity can be elicited many years after exposure to antigen, it is reasonable to suggest that some Tdh cells are very long lived.

When antigen is injected intradermally, some is taken up by langerhans cells, which then migrate to the draining lymph node under the influence of TNF α. Antigen also diffuses into nearby capillaries. Circulating Tdh cells recognize this antigen, adhere to capillary endothelial cells, and emigrate from the capillaries to encounter antigen. This initial emigration takes many hours to occur. IFN γ, TNF α and IL-1, all act on endothelial cells to cause increased expression of adherence molecules and MHC class II molecules as well as the release of chemotactic peptides. The increased MHC class II expression by endothelial cells may also cause a local increase in antigen presenting ability. Once the Tdh cells encounter antigen, they release vasoactive

factors such as serotonin as well as macrophage-activating cytokines, macrophage cytokines. The initial T-cell response may also generate a lymphokine that attracts basophils. Basophil-derived serotonin (in rodents) or histamine (in humans) enhances the migration of mononuclear cells into the lesion. The resulting increase in vascular permeability and opening up of gaps between endothelial cells in capillaries permit more TDH cells to migrate from the blood into the tissue. The chemotactic factors cause immigration of T cells that are not specifically sensitized for the inducting antigen. Macrophages also accumulate in the lesion and may be activated as a result of the release of IFN γ. Some of the tissue damage seen in intense delayed hypersensitivity reactions may be due to release of enzymes and reactive oxygen metabolites from these macrophages. The macrophages ingest and eventually destroy the injected antigen. This permits the tissues to return to normal.

Cells other than lymphocytes and macrophages also participate in the delayed hypersensitivity reactions. Thus neutrophils are found in the early stage of the reaction. In some species, basophils are prominent in the inflammatory reaction.

This type of reaction called Cutaneous Basophils Hypersensitivity can be transferred between animals with antibody, with purified B cells, or with T cells. CBH is therefore a heterogeneous phenomenon. CBH is observed in chickens in response to intradermal Rous sarcoma virus, in rabbits in response to schistosomes, and in humans in allergic contact dermatitis and renal allograft rejection.

However, these days a commercial assay is available in which IFN γ cytokine is quantified as readout for mycobacterial immunity, such as the Quantiferon$^{\mathrm{TM}}$ test. Although currently IFN γ is probably the best marker of mycobacterial immunity, and is indispensable for protection, mouse experiments have demonstrated that the magnitude of the IFN γ response may not correlate with vaccination-induced protection. The immune correlates of human vaccination induced or post-infectious protection against TB disease are not known, but future studies will hopefully shed light on this important area.

1.10.2.2 Treatment of Tuberculosis

Drugs used for treatment of tuberculosis can be grouped into two categories:

1. First line drugs, which include R- Rifampicin (including some newer derivatives - Rifabutin, Rifapantine), H-Isoniazid, Z-Pyrazinamide, E-Etha-mbutol, and SM-Streptomycin.

2. Second line drugs, which include: Fluoroquinolones (Ofloxacin, Ciproflox-acin, Sparfloxacin), Macrolides (Clarithromycin), Ethionamide, Cycloserine, Capreomycin, Thiocetazone, Para-amino salicylic acid, and Aminoglycosides (Amikacin, Kanamycin)

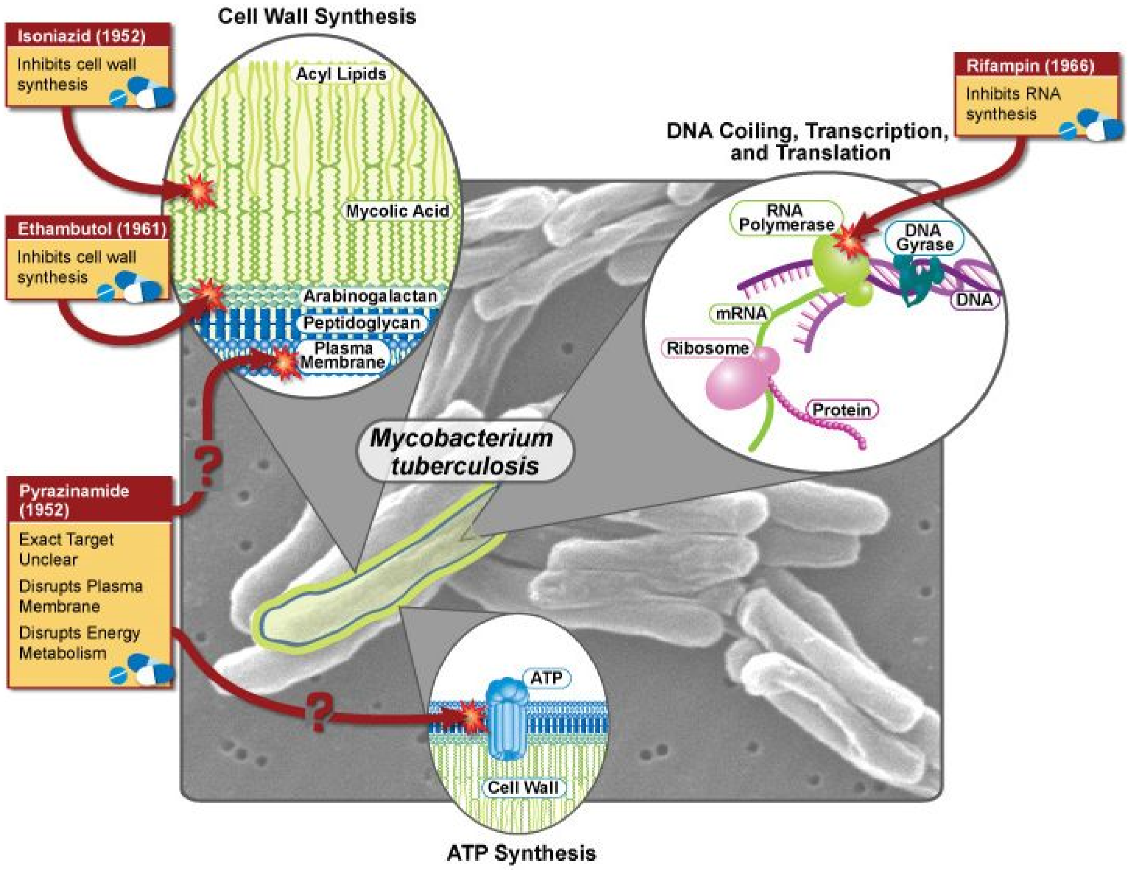


Figure 28: Tuberculosis, which results from an infection with Mycobacterium tuberculosis, can usually be cured with a combination of first-line drugs taken for several months. Shown here are the four drugs in the standard regimen of first-line drugs and their modes of action. Also shown are the dates these four drugs were discovered.
$*$ *Source* : *www3.niaid.nih.gov*

Active TB is treated with a combination of medications along with first line drugs. Four drugs (R- Rifampicin, H-Isoniazid, Z-Pyrazinamide and E-Ethambutol) are often taken for the first two months of therapy to help kill any potentially resistant strains of bacteria. Then the number is usually reduced to two drugs (H, R) for the remainder of the treatment based on drug sensitivity testing that is usually available by this time in the course. Streptomycin, a drug that is given by injection, may be used as well, particularly when the disease is extensive and/or the patients do not take their oral medications reliably (termed 'poor compliance'). Treatment usually lasts for many months and sometimes for years.

All these agents interfere with enzymes involved in cell wall biosynthesis (isoniazid, ethambutol, and ethionamide), protein synthesis (streptomycin, other aminoglycosides, and macrolides), transcription (rifampin), or DNA replication (quinolones). But multi drug resistant TB (MDR TB) cases are resistant to first line drugs (mainly

isoniazid and rifampicin) hence second line drugs are used. Second line drugs have either low anti tubercular efficacy or high toxicity or both and are used in special circumstances only. These include amikacin, kanamycin and capreomycin.

Bacille Calmetter Guérin, also known as BCG, is a vaccine given throughout many parts of the world. It is derived from an atypical mycobacterium but offers some protection from developing active tuberculosis, especially in infants and children.

Cellular targets of major anti-mycobacterials

Drug	Cellular target	Gene	Gene product/Functional role
Rifampin	Nucleic acids	*rpoB*	β-subunit of RNA polymerase/ transcription
Streptomycin	Protein synthesis	*rpsL*	Ribosomal protein S12/translation
		rrs	16S rRNA/translation
Pyrazinamide	Unknown	*pncA*	Pyrazinamidase-nicotinamidase/activation of prodrug
Ethambutol	Cell wall	*embB*	Arabinosyl transferase/arabinan polymerisation
Fluoroquinolones	Nucleic acids	*gyrA*	DNA gyrase subunit/DNA replication
Isoniazid	Cell wall	*katG*	Catalase-peroxidase/activation of prodrug
		inhA	enoyl-acyl carrier protein reductase/ mycolic acid biosynthesis
		kasA	β-ketoacyl acyl carrier protein synthase/ mycolic acid biosynthesis
		oxyR-ahpC	Alkylhydroperoxide reductase/unknown

Table 6: Mechanism of action of antibiotics.
$\star$ *Source : https : /.../lessons/antibiotic/$_t$able1.gif/*

1.10.2.2.1 Immunology of Tuberculosis

The immune system is a remarkably versatile defence system that has evolved to protect us from invading pathogenic microorganisms and cancer. It is able to appreciate the subtle differences between different pathogens along with having the power to acknowledge difference between self and non self. Hence, it is the second exposure to the same antigen that induces a memory response and evokes a more rapid and heightened immune reaction that servers to eliminate the pathogen and prevent the disease. The body has three lines of defence.

The first line of defence includes barriers at portals of entry which are primarily

inborn and nonspecific whereas the second line is replete with protective cells and chemicals.

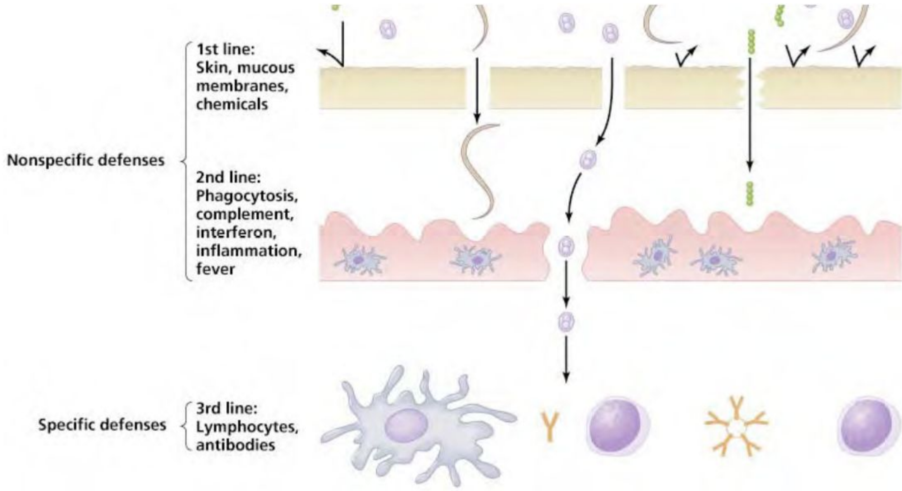


Figure 29: Different lines of defences.
⋆ *Source : www.merritt.edu/Projects/40279/Bio3_ch_15post.pdf*

The non specific components are innate whereas the specific components are acquired which accounts for the differential susceptibilities towards different diseases in a same or different situation. It is this adaptive specific immune system which requires co-operation between lymphocytes and antigen presenting cells namely macrophages, dendritic cells and monocytes. Therefore contraction of a disease only occurs when these portals have been broken or when body is not able to discriminate between self and non-self.

All blood cells arise from Hematopoietic stem cells (HSC). Stem cells have the power to differentiate into other cell types as they are pluripotent and self renewing. HSC give rise to lymphoid and myeloid progenitors. All lymphoid cells descend from lymphoid progenitor cells and all cells of the myeloid lineage arise from myeloid progenitors. These cells play a significant role in mounting an immune response.

M. Tuberculosis which is an intracellular pathogen after infecting the humans is readily engulfed by the macrophages because M. tuberculosis is an exogenous antigen. Macrophages and other phagocytic cells have evolved a system of receptors capable of recognizing molecular patterns expressed on the surface of the pathogens (PAMPs) which are conserved and clearly distinguishable from self patterns. They survive and multiply within the normal macrophage. This is an environment free of antibody and as a result humoral immune response is ineffective against these organisms therefore it is said that in this case immune response is cell mediated. Cell mediated immune

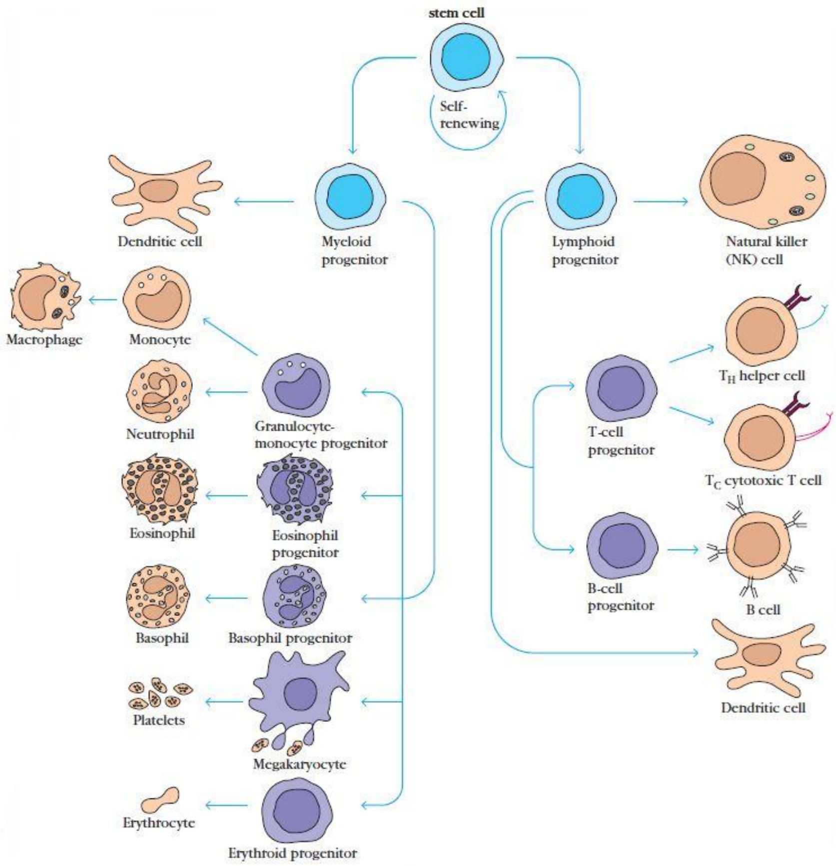


Figure 30: Differentiation of Haematopoietic Stem cells.
$*$ *Source* : *Kuby*

response involves T lymphocytes which although arise in Bone marrow but migrate to Thymus for maturation. During its maturation T-cell expresses an antigen binding molecule on its membrane called T-cell receptor (TCR). This receptor can recognise antigen bound to class II MHC molecules, which are expressed by antigen presentation cells (MHC Restriction).

Therefore for M. tuberculosis cell mediated particularly Th1 response is very significant as it produces a cytokine profile that supports inflammation and activates certain T cells and macrophages whereas Th2 activates mainly B cells and immune responses that depend upon antibodies is not very proficient. These subsets also differ in the types of cytokines produced. When a naive T cell encounters an antigen coupled to an MHC molecule on the cell, the T cell proliferates and differentiates into Th and Tc and memory cells. T cells that express the membrane glycoprotein molecule CD4 are restricted to recognizing antigen bound to class II MHC molecules, whereas T cells expressing CD 8, a dimeric membrane glycoprotein, are restricted to recognition of antigen bound to class I MHC molecules.

Th1	**Th2**
IL-12 induced differentiation	IL-14 induces differentiation
Cytokine production	Cytokine production
Interferon γ	Interleukin-4
Interleukin-2	Interleukin-5
	Interleukin-3
Intracellular pathogens	Extracellular pathogens
Macrophage activation	B cell activation, and IgE
	Eosinophil responses
Delayed type hypersensitivity	Immediate type hypersensitivity

Table 7: Different cytokines are produced by T helper cells.

Thus the expression of CD 4 versus CD 8 corresponds to the MHC restriction of the T cell. In general, expression of CD 4 and of CD 8 also defines two major functional subpopulations of T lymphocytes. CD4- T cells generally function as T helper (TH) cells and are class-II restricted; CD8-T cells generally function as T cytotoxic (TC) cells and are class-I restricted. Thus the ratio of TH to TC cells in a sample can be approximated by counting the number of CD4 and CD8 T cells. This ratio is approximately 2:1 in normal human peripheral blood, but it may be altered by immunodeficiency diseases, autoimmune diseases, and other disorders.

After a TH cell recognizes and interacts with an antigen that is an MHC class II molecule complex, the cell is activated - it becomes an effector cell that secretes various growth factors known collectively as cytokines. The secreted cytokines play an important role in activating B cells, TC cells, macrophages, and various other cells that participate in the immune response. Differences in the pattern of cytokines produced by Activated TH cells result in different types of immune response. Under the influence of TH-derived cytokines, a TC cell that recognises an antigen - MHC class I molecule complex proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL).

In contrast to the TC cell, the CTL generally does not secrete many cytokines and instead exhibits cell-killing or cytotoxic activity. The CTL has a vital function in monitoring the cells of the body and eliminating any that display antigen, such as virus-infected cells, tumour cells, and cells of a foreign tissue graft. Cells that display foreign antigen complexed with a class I MHC molecules are called altered self-cells; these are targets of CTLs.

T cells recognize lipoidal compounds such as glycolipids and phospholipids when presented as complexes with molecules that are like MHC molecules. These are members of the CD1 family. In case of Mycobacterium tuberculosis, T cells effectively recognise the antigen molecules complexed with CD1. This is very significant in terms of presentation of nonpeptide antigens which follow a signalling pathway whose mecha-

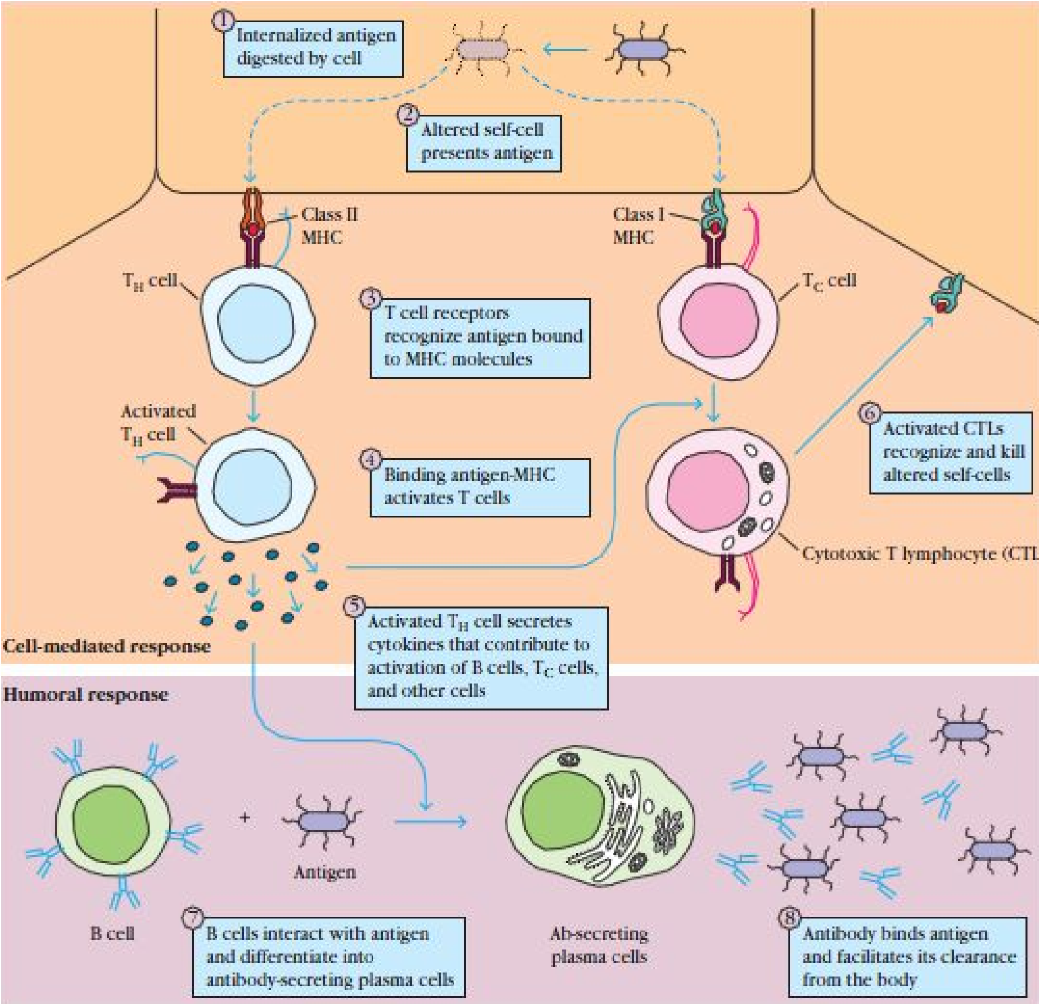


Figure 31: Humoral and cell mediated responces.
$\star$ *Source* : *Kuby*

nism is not known till date.

When few small droplets of bacilli are inhaled by a human, these bacilli are then ingested by the alveolar macrophages where they are able to survive and multiply intracellularly by inhibiting the formation of phagolysosomes. When infected macrophages lyse, CD4T cells are activated within 2-6 weeks after infection and tissue damage occurs and infiltration of large number of activated macrophages. CD4 T-cell activity is the basis for the tuberculin test.

Cell wall off the organism inside a granulomatous lesion called a tubercle. A tubercle consists of a few small lymphocytes and a compact collection of activated macrophages, which sometimes differentiate into epithelioid cells or multinucleated giant cells. The massive activation of macrophages that occurs within tubercles often results in the concentrated release of lytic enzymes. These enzymes destroy nearby

healthy cells, resulting in circular regions of necrotic tissue, which eventually form a lesion with a caseous (cheese like) consistency. As these caseous lesions heal, they become calcified and are readily visible on x-rays, where they are called Ghon complexes. Because the activated macrophages suppress proliferation of the phagocytosed bacilli, infection is contained.

Cytokines produced by CD4+ T cells (TH1 subset) play an important role in the response by activating macrophages, so that they are able to kill the bacilli or inhibit their growth. The role of IFN in the immune response to mycobacteria has been demonstrated with knockout mice lacking IFN. These mice died when they were infected with an attenuated strain of mycobacteria (BCG), whereas IFN + normal mice survive. In studies related to high levels of IL-12 in the pleural effusions of

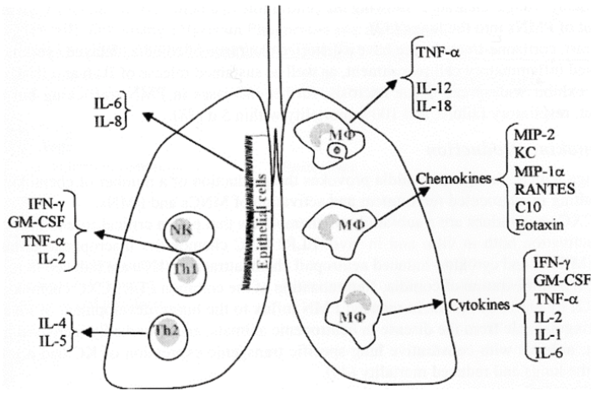


Figure 32: Secretion of cytokines by the macrophages.
$\star$ *Source : dms.dartmouth.edu/aitrp/shortterm/pdf/4$_r$espiratory$_t$ract.pdf*

tuberculosis patients, The high levels of IL-12, produced by activated macrophages, are not surprising, given the decisive role of IL-12 in stimulating TH1-mediated responses. In mouse models of tuberculosis, IL-12 has been shown to increase resistance to the disease. Not only does IL-12 stimulate development of TH1 cells, but it also may contribute to resistance by inducing the production of chemokines that attract macrophages to the site of infection. When IL-12 is neutralized by antibody to IL-12, granuloma formation in tuberculosis mice is blocked. The CD4+ T cell mediated immune response mounted by the majority of people exposed to M. tuberculosis thus controls the infection and later protects against reinfection. However, about 10% of individuals infected with M. tuberculosis follow a different clinical pattern: the disease progresses to chronic pulmonary tuberculosis or extrapulmonary tuberculosis. This progression may occur years after the primary infection. In this clinical pattern, accumulation of large concentrations of mycobacterial antigens within tubercles leads to extensive and continual chronic CD4+ T-cell activation and ensuing macrophage activation. The resulting high concentrations of lytic enzymes cause the necrotic caseous lesions to liquefy, creating a rich medium that allows the tubercle bacilli to proliferate extracellularly. Eventually the lesions rupture, and the bacilli disseminate

in the lung and/or are spread through the blood and lymphatic vessels to the pleural cavity, bone, urogenital system, meninges, peritoneum, or skin.

1.11 Defense strategies of Mycobacteria Tuberculosis

- Mycobacterium avoids being killed by actively blocking both, recruitment of ATP proton pumps containing vacuoles and lysosome fusion. Mtb. Phagosome pH stays high and free of lysosomal contents.
- Mtb. May inhibit vesicular trafficking of NADPH, Inos and ATP proton pumps containing vacuoles and lysosomes to the mycobacterial phagosome as a means to evade repiratory burst of reactive oxygen and nitrogen radicals. Respiratory burst occurs in phagocytic cells namely macrophages and neutrophils to eradicate infection
- Mycobacterium produces potent iron binding proteins called siderophores which have high affinity for iron so they withdraw it from the serum proteins and make it available to the bacteria. In diseases wherein serum iron levels are elevated like in haemolytic anaemias, individuals may be susceptible to overwhelming bacterial infections.

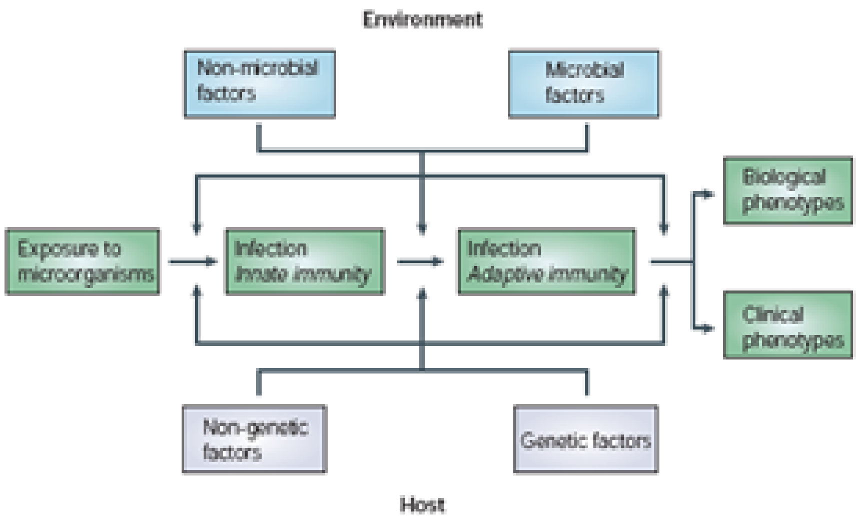


Figure 33: A Schematic Showing the Stages of the host-environment interaction in the course of infection.

$\star$ *Source* : *NatureReviewImmunology*2004

1.12 Genetic aspect of infectious disease

The sequence of the human genome has been determined. This 'reference' human genome sequence does not match in everyone exactly; in fact, it does not correspond to the genome of any one person, but is just a framework sequence for the human genome, patched together from different sources. Real individual genomes will be different from this reference sequence in a variety of ways: there is variation in the exact sequence, due to single base changes, but also variation in the general structure of the genome, due to variation in the numbers of repeat regions, including even variation in whether a sequence is present or absent (or, if present, how many times). Although these differences are often neutral (irrelevant to a person's health and other characteristics) genome variation can also influence disease and this is where the concept of mapping and association gains importance. It is through Genetic mapping

that we get to know the loci of a particular gene and also its association with other genes. Genetic mapping focuses on different genetic testing techniques which make use of particular DNA marker techniques depending on the type of experimental study. These DNA markers help understand DNA polymorphisms. These are also used in DNA typing for identifying individuals, tracking the course of virus and bacterial epidemics, studying human population history, and improving cultivated plants and domesticated animals, as well as for the genetic monitoring of endangered species and for many other purposes. These DNA markers with polymorphisms are the genetic

variations, in the form of multiple alleles of genes, between individuals. These markers include various types. Some of the commonly used are : -

- Single Nucleotide Polymorphism (SNP) An SNP is a single base pair mutation at a specific locus, usually consisting of two alleles (where the rare allele frequency is = 1%). For example, some DNA molecules may have a T/A base pair at a particular nucleotide site, whereas other DNA molecules in the same population may have a C/G base pair at the same site. This difference constitutes a SNP. The SNP at a locus is biallelic, for which there could be three genotypes among individuals in the population: homozygous with T/A at the corresponding site in both homologous chromosomes, homozygous with C/G at the corresponding site in both homologous chromosomes, or heterozygous with T/A in one chromosome and C/G in the homologous chromosome. In the human genome, any two randomly chosen DNA molecules are likely to differ at one SNP site about every 1000 - 3000 bp in protein coding DNA and at about one SNP site every 500 - 1000 bp in noncoding DNA.

- Restriction Fragment Length Polymorphism (RFLP) RFLP is a variation in the DNA sequence of a genome which results due to the presence or absence of a restriction site by a SNP. The basic technique for detecting RFLPs involves the fragmentation of genomic DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific recognition sequence for a given restriction enzyme occurs, in a process known as a restriction digestion. Because RFLPs

change the number and size of DNA fragments produced by digestion with a restriction enzyme, they can be detected by gel electrophoresis. Analysis of RFLP variation in genomes is a vital tool in genome mapping and genetic disease analysis. RFLP analysis is also the basis for early methods of Genetic fingerprinting, useful in the identification of samples retrieved from crime scenes, in the determination of paternity, and in the characterization of genetic diversity or breeding patterns in animal populations.

- Simple Sequence Length Polymorphism (SSLP) These correspond to the variability in the length of repetitions of DNA. STRs are repeated numerous times in a head-tail manner. STRPs that are present at different loci may differ in the sequence and length of the repeating unit, as well as in the minimum and maximum number of tandem copies that occur in DNA molecules in the population. The polymorphisms in STRs are due to the different number of copies of the repeat element that can occur in a population of individuals. STRs are of the following major types depending upon the average size of the repeating units : -
  - Satellite DNA (100kb to several Mb in length) found in the centromeric heterochromatin of most chromosomes.
  - Minisatellite DNA (0.1-20kb in length) found in telomeres of all chromosomes.
  - Microsatellite DNA (less than 150bp) found in all chromosomes. A microsatellite is also called as a simple sequence length polymorphism (SSLP).

  †A minisatellite is also called as variable number of tandem repeats (VNTR). Because of their high degree of variation among people, STRPs are widely used in DNA typing (sometimes called DNA fingerprinting) to establish individual identity for use in criminal investigations etc.

- Single strand conformational polymorphism (SSCP) SSCP is defined as conformational difference of single stranded nucleotide sequences of identical length as induced by differences in the sequences under certain experimental conditions. This property allows distinguishing the sequences by means of gel electrophoresis, which separates different conformations. Double strands are almost identical for both alleles. After denaturation, single strands undergo a 3-dimensional folding and may assume different conformational state according to DNA sequence. Depending on this folded structure that the molecules have assumed, they may travel faster or slower on a gel, even though the number of nucleotides is the same. This implies the limitation of SSCP, since conformational states are subject to many experimental conditions and sequence differences may or may not be detected. It is mostly used to identify the heterozygous individuals.

- Copy number polymorphism (CNPs) CNP refers to the differences in the number of copies of a greater than 1Kb to Mb region of DNA or a gene, fully or

partially, in the genome of an individual. Like other types of genetic variation, some gene copy number variants have been identified to carry susceptibility or resistance to a disease. Copy number variations are invariably elevated in cancer cells.

The next step in genetic mapping is association mapping which helps to calculate the significance of the particular polymorphism. Before one could start with these above mentioned steps of genetic mapping one has to complete some of the primary steps which help in building the material for further analysis. These primary steps are : -

- Purification of genomic DNA from cell extract DNA is purified from cellular extract by organic solvent extraction and enzyme digestion. The organic extraction step mainly involves use of lysis buffer and providing phenol chloroform treatment. The main step is the quantification of the purified DNA which could be performed with a spectrophotometer. This helps in formulating the next steps in order to increase the efficiency of the experiment. Purification step involves the following -
  - Lysis buffer treatment; Helps break the cell wall for the release of genomic DNA and other cellular contents.
  - Phenol-chloroform treatment; Helps the protein content of the cellular extract to denature and pelleting down of this unwanted material after centrifugation.
  - Chilled ethanol treatment; Helps in the precipitation of nucleic acid content of the cell which can be easily separated later.

- Polymerase Chain Reaction
  The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by in vitro enzymatic replication. As PCR progresses, the DNA thus generated is itself used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies.

Depending upon the site concerned that is the presence or absence of restriction site, further techniques are performed. If the site concerned has a restriction site then RFLP is performed and if it has microsatellite repeats then Native PAGE is carried out and accordingly statistical calculations and analyses are performed.

1.12.1 Association mapping of infectious diseases

It has developed into one of the most dynamic research areas of human genetics. It focuses on identifying functional polymorphisms that predispose to complex diseases, such as Tuberculosis. Population-based approaches have exploited linkage disequilibrium (LD) between single nucleotide polymorphisms (SNPs) and disease predisposing loci. The utility of SNPs in association mapping is now well established and the interest in this field has been enhanced by the discovery of millions of SNPs across the genome (Maniatis 2007). Genomic studies are being carried out to understand the

inheritance of disease and their diagnosis to understand them in a better way. The inheritance of various traits occurs from generation to generation via chromosomal or mitochondrial DNA. DNA keeps accumulating changes due to mutations or recombination in the form of VNTRs, SNPs, and Insertions/Deletions etc. The variations in DNA contribute to the differences in the level of resistance and susceptibility to various diseases in individuals and population groups. Therefore, the studies to understand genetic predisposition, in complex diseases, like Tuberculosis, are expected to provide relevant information for disease management (Collins, Green et al. 2003) in future.

Part II

Review of Literature

2 Review

In case of infectious diseases, only a proportion of individuals who are exposed to a pathogen become infected and develop clinically evident disease. This inter-individual variability in susceptibility is determined by the combined effect of host proteins encoded by a series of genes that control the quantity and quality of host-parasite interaction as well as host immune responses. The identification of the most important host susceptibility/resistance genes therefore can give a better understanding of infectious disease pathogenesis. Several approaches can be used to map and identify a host infectious disease susceptibility/resistance gene such as mouse models, candidate gene approach, and genome-wide scanning. To date, several genes have been implicated in susceptibility/resistance to mycobacterial infection.

2.1 Mouse model

An approach to identify human disease resistance and susceptibility genes is to identify murine resistance/susceptibility genes. In this strategy, it is assumed that the basic pathology of the infectious disease is similar in the animal model and the human host. Consequently, orthologous genes in mouse and humans are assumed to be important for variable susceptibility/resistance to infection with the same pathogen. An example for a susceptibility gene that has been identified in the mouse is the 'natural resistance associated macrophage protein1' (Nramp1).

2.2 Candidate gene approach

Candidate genes are generally selected on the basis of their known or speculated relevance to disease pathogenesis and the presence of intragenic polymorphisms of possible biological significance. Candidate genes can also be derived based on experiments in mouse models of infectious diseases thereby exploiting the identification of murine resistance/susceptibility loci. The best example of this is same as of mouse model i.e, Nramp. Variants within a candidate gene can be analyzed in linkage studies (family studies) and/or in association studies (case-control studies), but in most cases, association studies are used to study the possible biological relevance of polymorphisms in specific candidate genes.

2.3 Total genome scanning

Genome-wide scan is an approach in which a large number of genetic markers like micro satellites, SNPs, etc. evenly spaced across the whole genome are being used for linkage analysis in families as well as in case control studies. The genome wide search has the advantage that no a priori knowledge of the structure or function of susceptibility genes is required. Hence, this approach provides the possibility of identifying genes that modulate susceptibility to infectious diseases that had previously not been suspected of playing such a biological role.

Susceptibility to tuberculosis has been suggested to be multifactorial. Though environmental and socio-economic factors are primarily related, numerous studies have

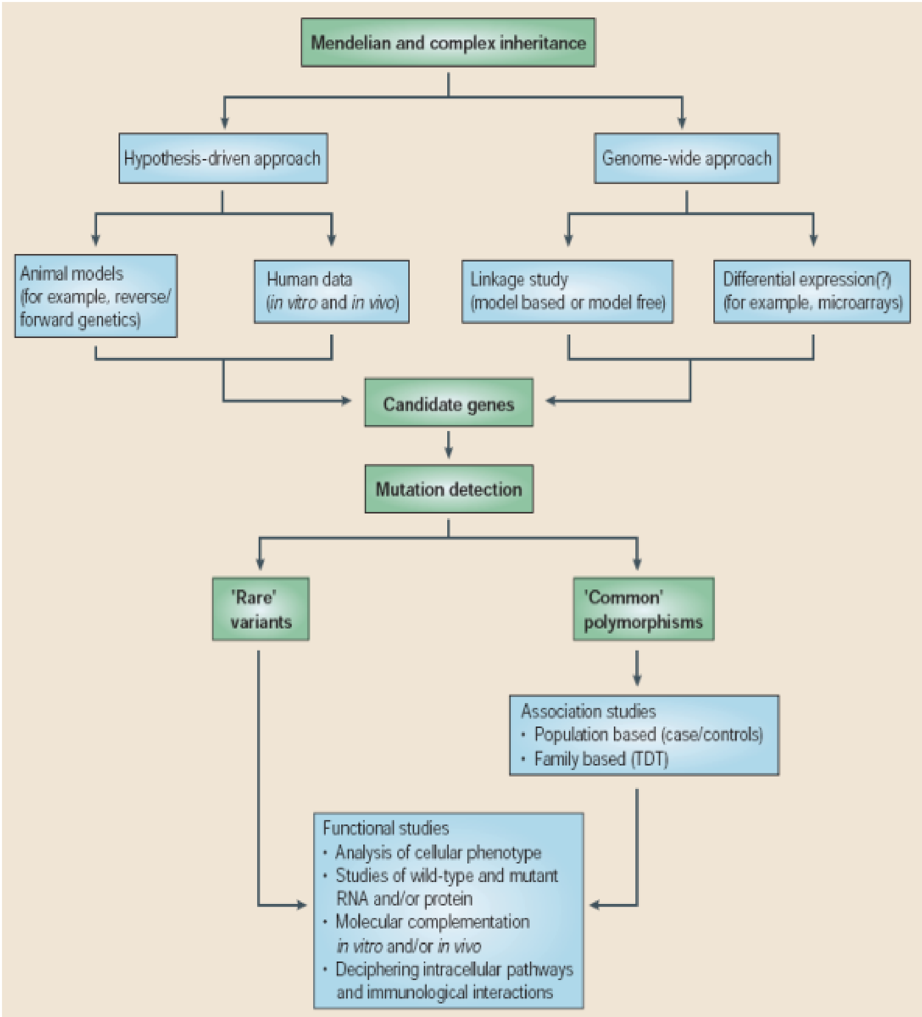


Figure 34: Methods in Human Genetics of infectious Diseases.
‡ *Source* : *Nature Review Immunology* 2004

emphasised the importance of host resistance and hereditary susceptibility. Susceptibility or resistance to tuberculosis may be under the control of the genetic make-up of an individual. The host genetic factors such as HLA (Human Leukocyte Antigen) and non-HLA genes may be associated with susceptibility or resistance to tuberculosis. Finding out of HLA and non-HLA genes/gene products (antigens) which are associated with susceptibility or resistance to tuberculosis serve as efficient genetic markers to understand the development of the disease. Moreover, studying the role of these markers in the immune mechanism underlying susceptibility or resistance to tuberculosis are useful for understanding the immunopathogenesis of the disease. Recently, human genome analysis has revealed several candidate HLA (class 1 as HLA - A, B, C and class 2 as - DR -DQ and -DP) and non HLA genes which occur in polymorphic forms. Such polymorphic genes have been shown to be associated with susceptibility to a number of infectious diseases like tuberculosis and some non-infectious disease.

Some of these non-HLA genes are SLC11A1 encoding for divalent cation transporters, NOS gene encoding for Nitric oxide synthase, VDR encoding for Vitamin D receptor, SP110 encoding for Transcription factor, MBL encoding for Mannose binding Lectin and P2RX7 encoding for ATP receptor etc.

Locus	Description
HLA-DRB1	HLA class II molecule
SLC11A1 (NRAMP1)	Divalent cation transporter
INFG	Interferon gamma cytokine
VDR	Vitamin D receptor
SP110	Transcription factor
IL8	Interleukin-8 cytokine
UBE3A	Ubiquitin ligase
MAL/TIRAP	TLR signaling pathway adaptor
P2RX7	ATP receptor
IL10	Interleukin-10 cytokine
DC-SIGN	C-type lectin receptor on dendritic cells
SP-A	Surfactant protein
CR1	Complement receptor
CCL2	Monocyte chemoattractant protein-1 chemokine
IL12RB1	Interleukin-12 cytokine receptor chain
INFGR1	Interferon gamma cytokine receptor chain
CR1	Complement receptor, CD35
TLR2	Toll-like receptor-2
MBL2	Mannose-binding lectin

Table 8: Some loci implicated in Tuberculosis susceptibility.
† *Source* : *Annual Review of Genetics*, 2006.

2.3.1 Interferon γ

Interferon γ's CA repeat polymorphism is present on chromosome 12q24.1 having a vast genome size of 5.4 Kb and it spans four exons with an amplicon size of 144 bp. Interferon γ is the most important cytokine providing resistance to mycobacterial diseases and variations within interferon γ gene are associated with tuberculosis susceptibility as it plays an essential role in the activation of cell mediated immunity required for the elimination of pathogens. Mycobacterium tuberculosis elicits T cell responses and production of IFN γ that is essential for control of infection, but is insufficient to eradicate the pathogen, which persists in long-term chronic infection, harboured by macrophages. Mice that are deficient in IFN γ develop fatal, disseminated mycobacterial disease. Similarly, human mutations linked to IFN γ signalling are associated with disseminated infection after BCG vaccination and increased susceptibility to mycobacterial infection.

T cells are basically responsible for cell mediated immunity, therefore it is essential

to discuss about T cells. Antigen recognition by T cells is different from recognition by B cells in terms of MHC restriction i.e., a T cell has an antigen binding receptor called TCR which is only able to recognise an antigen if it is coupled to an MHC molecule. In most cases, both the maturation of progenitor T cells in the thymus and the activation of mature T cells in the periphery are influenced by the involvement of MHC molecules. The potential antigenic diversity of the T-cell population is reduced during maturation by a selection process that allows only MHC-restricted and nonself-reactive T cells to mature. The final stages in the maturation of most T cells proceed along two different developmental pathways which generate functionally distinct CD4+ and CD8+ subpopulations that exhibit class II and class I MHC restriction, respectively.

Activation of mature peripheral T cells begins with the interaction of the T-cell receptor (TCR) with an antigenic peptide displayed in the groove of an MHC molecule. Although the specificity of this interaction is governed by the TCR, its low avidity signifies the involvement of co receptors and other necessary membrane molecules that strengthen the TCR-antigen-MHC interaction and transduce the activating signal. Activation leads to the proliferation and differentiation of T cells into various types of effector cells and memory T cells.

The central event in generation of both humoral and cell-mediated immune responses is the activation and clonal expansion of Th cells. TH cell activation is initiated by interaction of the TCR-CD3 complex with a processed antigenic peptide bound to a class II MHC molecule on the surface of an antigen-presenting cell. This interaction and the resulting activating signals also involve various accessory membrane molecules on the TH cell and the antigen-presenting cell, like an antigen nonspecific co-stimulatory signal which is provided primarily by interactions between CD28 on the T cell and members of the B7 family on the APC. Interaction of a TH cell with antigen initiates a cascade of biochemical events that induces the resting TH cell to enter the cell cycle, proliferating and differentiating into memory cells or effector cells. Many of the gene products that appear upon interaction with antigen can be grouped into one of three categories depending on how early they can be detected after antigen recognition:

Immediate genes, expressed within half an hour of antigen recognition, encode a number of transcription factors, including c-Fos, c-Myc, c-Jun, NFAT, and NF-κB.

Early genes, expressed within 1-2 h of antigen recognition, encode IL-2, IL-2R (IL-2 receptor), IL-3,IL-6, IFN-γ, and numerous other proteins.

Late genes, expressed more than 2 days after antigen recognition, encode various adhesion molecules. These profound changes are the result of signal-transduction pathways that are activated by the encounter between the TCR and MHC-peptide complexes.

If a naive T cell recognizes an antigen-MHC complex on an appropriate antigen-

presenting cell or target cell, it will be activated, initiating a primary response. About 48 hours after activation, the naive T cell enlarges into a blast cell and begins undergoing repeated rounds of cell division. Activation depends on a signal induced by engagement of the TCR complex and a co-stimulatory signal induced by the CD28-B7 interaction. These signals trigger entry of the T cell into the G1 phase of the cell cycle and, at the same time, induce transcription of the gene for IL-2 and the α chain of the high-affinity IL-2 receptor. In addition, the co-stimulatory signal increases the half-life of the IL-2 mRNA. The increase in IL-2 transcription, together with stabilization of the IL-2 mRNA, increases IL-2 production by 100-fold in the activated T cell. Secretion of IL-2 and its subsequent binding to the high-affinity IL-2 receptor induces the activated naive T cell to proliferate and differentiate. T cells activated in this way divide 2-3 times per day for 4-5 days, generating a large clone of progeny cells, which differentiate into memory or effector T-cell populations.

The various effector T cells carry out specialized functions such as cytokine secretion and B-cell help (activated CD4+ TH cells) and cytotoxic killing activity (CD8+ CTLs). Effector cells are derived from both naive and memory cells after antigen activation. Effector cells are short-lived cells, whose life spans range from a few days to a few weeks. The effector and naive populations express different cell-membrane molecules, which contribute to different recirculation patterns. CD4+ effector T cells form two subpopulations distinguished by the different panels of cytokines they secrete. One population, called the TH1 subset, secretes IL-2, IFN-γ, and TNF-β. The TH1 subset is responsible for classic cell-mediated functions, such as delayed-type hypersensitivity and the activation of cytotoxic T lymphocytes. The other subset, called the TH2 subset, secretes IL-4, IL-5, IL-6, and IL-10. This subset functions more effectively as a helper for B-cell activation.

IFN γ is a defining cytokine of the TH1 subset. Cytokines are basically low molecular weight regulatory proteins or glycoproteins secreted by various cells in the body in response to a number of stimuli. These assist in regulating the development of immune effector cells and some cytokines also possess direct effector functions of their own. Binding of a given cytokine to responsive target cells generally stimulates increased expression of cytokine receptors and secretion of other cytokines, which affect other target cells in turn.

IFN-γ, activates macrophages, stimulating these cells to increase microbicidal activity, up-regulate the level of class II MHC, and secrete cytokines such as IL-12, which induces TH cells to differentiate into the TH1 subset. IFN-γ secretion by TH1 cells also induces antibody-class switching to IgG classes (such as IgG2a in the mouse) that support phagocytosis and fixation of complement. TNF-$and IFN-\gamma$ are cytokines that mediate inflammation, and it is their secretion that accounts for the association of TH1 cells with inflammatory phenomena such as delayed hypersensitivity. TH1 cells produce IL-2 and IFN-γ cytokines that promote the differentiation of fully cytotoxic TC cells from CD8+ precursors. This pattern of cytokine production

makes the TH1 subset particularly suited to respond to viral infections and intracellular pathogens. Finally, IFN-γ inhibits the expansion of the TH2 population which stimulates eosinophil activation and differentiation, provides help to B cells.

2.3.1.1 Interferon receptor

IFN-γ must first bind to specific receptors expressed on the membrane of responsive target cells. Because these receptors are expressed by many types of cells, therefore IFN γ can affect a diverse array of cells.

Interferon γ belongs to class II cytokine receptors which possess conserved amino acid

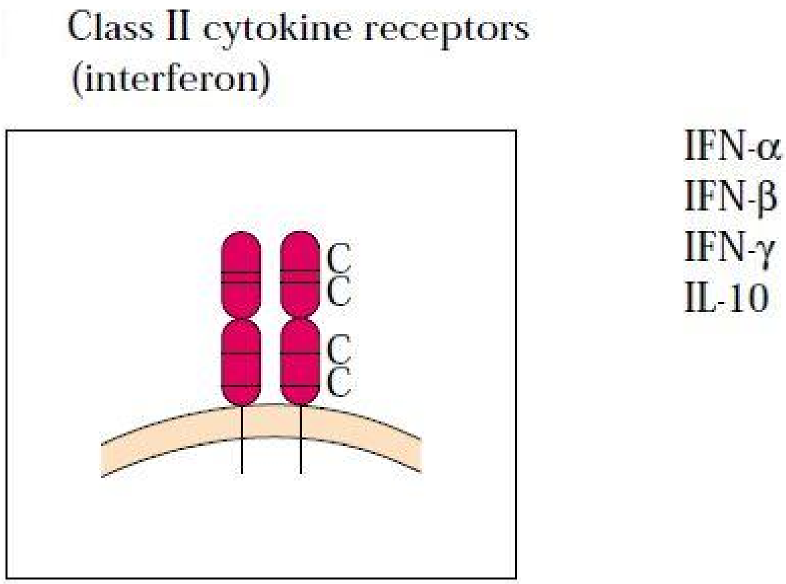


Figure 35: Interferon receptor.
† *Source* : *Kuby*

sequence motifs in the extracellular domain consisting of four positionally conserved cysteine residues (CCCC) but lack intrinsic signalling motifs like intrinsic tyrosine kinase domains. Initially only three interferons: α, β and γ were thought to be the ligands for these receptors, however later IL-10 was also included. Interferon Receptors lack enzymatic activity but attract and activate cytoplasmic enzymes that act on downstream proteins either by directly converting them to gene regulating proteins or by activating a cascade of enzymes that finally activates a gene regulator. The JAK-STAT system exemplifies the first mechanism and TLR4 (Toll) signalling system through which mammals detect the bacterial lipopolysaccharide (LPS), a potent toxin.

2.3.1.1.1 Following represents the main points regarding interferon γ receptor

The cytokine receptor is composed of separate subunits, an α chain required for cytokine binding and for signal transduction and a β chain necessary for signaling but with only a minor role in binding. Different inactive protein tyrosine kinases are associated with different subunits of the receptor.

The α chain of the receptor is associated with a novel family of protein tyrosine kinases, the Janus kinase (JAK)* family. The association of the JAK and the receptor subunit occurs spontaneously and does not require the binding of cytokine. However, in the absence of cytokine, JAKs lack protein tyrosine kinase activity. Cytokine binding induces the association of the two separate cytokine receptor subunits and activation of the receptor-associated JAKs. The ability of IFN-γ, which binds to a class II cytokine receptor, to bring about the association of the ligand-binding chains of its receptor has been directly demonstrated by x-ray crystallographic studies.

Activated JAKs create docking sites for the STAT transcription factors by phosphorylation of specific tyrosine residues on cytokine receptor subunits. Once receptor associated JAKs are activated, they phosphorylate specific tyrosines in the receptor subunits of the complex. Members of a family of transcription factors known as STATs (signal transducers and activators of transcription) bind to these phosphorylated tyrosine residues.

Specific STATs play essential roles in the signaling pathways of a wide variety of cytokines. The binding of STATs to receptor subunits is mediated by the joining of the SH2 domain on the STAT with the docking site created by the JAK-mediated phosphorylation of a particular tyrosine on receptor subunits. After undergoing JAK-mediated phosphorylation, STAT transcription factors translocate from receptor docking sites at the membrane to the nucleus, where they initiate the transcription of specific genes.

While docked to receptor subunits, STATs undergo JAK-catalyzed phosphorylation of a key tyrosine. This is followed by the dissociation of the STATs from the receptor subunits and their dimerization. The STAT dimers then translocate into the nucleus and induce the expression of genes containing appropriate regulatory sequences in their promoter regions.

Subsequently, the activated JAKs phosphorylate various tyrosine residues, resulting in the creation of docking sites for STATs on the receptor and the activation of the one or more STAT transcription factors. The phosphorylated STATs dimerize and translocate to the nucleus, where they activate transcription of specific genes.

IFN γ activates macrophages to kill diverse intracellular pathogens, but does not activate human macrophages to kill virulent Mycobacterium tuberculosis. It is often reported that this is due to inhibition of IFN γ signalling by M. tuberculosis. M.

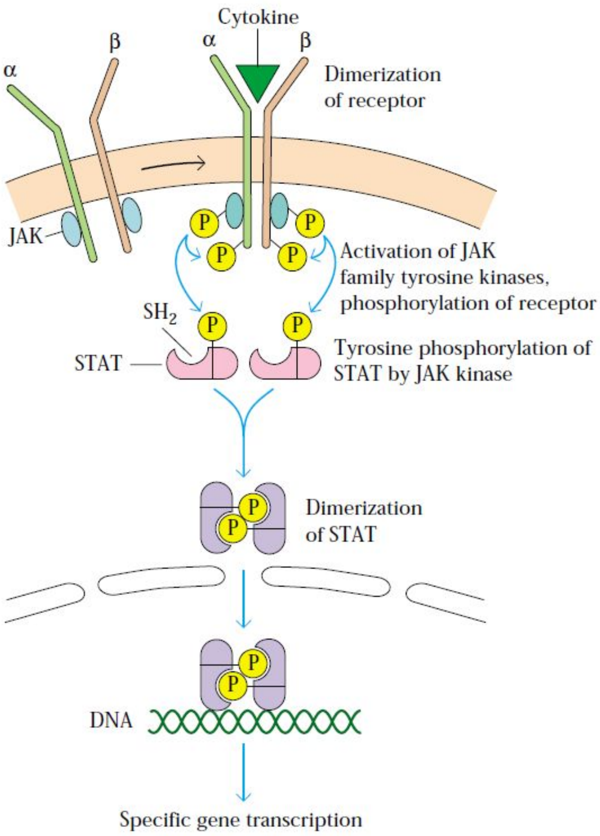


Figure 36: Binding of a IFN gamma induces dimerization of the receptor subunits, which leads to the activation of receptor-subunit-associated JAK tyrosine kinases by reciprocal phosphorylation.

† *Source* : *Kuby*

tuberculosis infection of human macrophages blocks several responses to IFN γ. The inhibitory effect of M. tuberculosis is directed at the transcription of IFN γ responsive genes but does not affect proximal steps in the Janus kinase-STAT pathway.

This is evident because STAT1 α tyrosine and serine phosphorylation, dimerization, nuclear translocation, and DNA binding are intact in M. tuberculosis-infected cells. Therefore it is said that M. tuberculosis evades the human immune response by inhibiting the IFN γ signalling pathway.

2.3.2 Vitamin D receptor

Immunity to tuberculosis is cell mediated, the intercellular interaction being mediated by cytokines which play an important role in determining the disease outcome. Generally Th1 cytokines are considered to be protective against tuberculosis and th2 cytokines makes the disease severe. Differentiation of T cells into Th1 or Th2 cell type depends on the local cytokine environment. Interleukin-12 favours Th1 development whereas Interleukin-4 is a potent inducer of Th2 cell differentiation.

At the site of tuberculous lesion, there is a selective concentration of Th1 and not Th2 cells. However, this does not confer protection, because the Th1 response is generally depressed in tuberculosis and this could be due to the T cell anergy or enhanced production of immunosuppressive cytokines or other factors. The proinflammatory cytokines, IL-8 and IL-6, released early from the macrophages are important in the initial immune defense against tuberculosis. Interleukin-8, which is released by activated alveolar macrophages upon phagocytosis of M. tuberculosis, is a potent chemotactic factor for neutrophils and T lymphocytes and IL-6 is involved in stimulating early Interferon γ (IFN-γ) production, but has also been shown to suppress T cell responses. The Th1 inducing cytokine, IL-12 is secreted following phagocytosis of M. tuberculosis by macrophages and dendritic cells, and is crucial for resistance against the infection.

Interferonγ, a classical Th1 cytokine has enhanced antimycobacterial properties and is essential for macrophage activation. Although the levels of IFN-γ are higher at the site of infection, the macrophages fail to respond to it due to some host genetic defects or effects mediated by M. tuberculosis. The anti-inflammatory cytokines, Transforming growth factor-b (TGF-b) and IL-10 have macrophage deactivating properties and have been shown to suppress Th1 responses in TB patients. Interleukin-4 (IL-4) and IL-5 are Th2 type of cytokines, and IL-4 has been shown to down regulate protective Th1 responses

The secosteroid hormone vitamin D, its receptor (VDR), and the metabolizing enzymes involved in the formation of the biologically active form of the hormone, calcitriol, together are major players in the vitamin D endocrine system. VDR is synthesized in monocytes and activated T and B lymphocytes whereas its ligand 1, 25 dihydroxy vitamin D is produced in kidney and by activated monocytes and macrophages, in particular in granuloma. Located in the 12q13 region, this system plays an important role in skeletal metabolism, including intestinal calcium absorption, but has also been shown to play an important role in other metabolic pathways, such as those involved in the immune response and cancer Haussler, et al. (1998). In the immune system, for example, vitamin D promotes monocyte differentiation and inhibits lymphocyte proliferation and secretion of cytokines, such as interleukin 2 (IL2), interferon-γ, and IL12. In several different types of cancer cells, vitamin D has been shown to have anti proliferative effects. Most of the biological activities of 1,25(OH)2D3 are mediated by a high-affinity receptor that acts as a ligand-activated transcription factor. The major steps involved in the control of gene transcription by the vitamin D receptor (VDR) include ligand binding, heterodimerization with retinoid X receptor (RXR), binding of the heterodimer to vitamin D response elements (VDREs), and recruitment of other nuclear proteins into the transcriptional preinitiation complex.

Thus, genetic alterations of the VDR gene could lead to important defects on gene activation, affecting calcium metabolism, cell proliferation, immune function, etc., which could be explained by changes in the protein sequence. For instance, delete-

rious mutations in the VDR gene cause 1,25-dihydroxivitamin D resistant rickets, a rare monogenetic disease Sone et.al. (1990). More subtle sequence variations (polymorphisms) in the VDR gene also occur more frequently, but the significance of it has not been systematically analyzed and the effects on the VDR protein levels and function are unknown. A polymorphism is a genetic variant that appears in at least 1% of the population. These changes can occur in non-coding parts of the gene (introns), so they would not be seen in the protein product. Changes in these regulatory parts of the gene would then affect the degree of expression of the gene, and thus the levels of the protein. For instance, changes in the 5'-promoter of the VDR gene can affect mRNA expression patterns and levels, while 3' untranslated region (UTR) sequence variations can affect the mRNA stability and protein translation efficiency. However, the changes can take place in exonic parts of the DNA, then leading to changes in the protein sequence. Nonetheless, changes in exonic sequences of the DNA which do not alter the protein structure are also possible, and are called synonymous polymorphisms.

2.3.2.1 VDR gene polymorphism

Information on the existence of VDR polymorphisms so far has come from analysis of only limited areas in the gene and by using rather less-sensitive techniques to find polymorphisms, such as screening with different restriction enzymes for polymorphic banding patterns in Southern blot hybridization experiments. Examples of these include the Tru9I, TaqI, BsmI, EcoRV and ApaI. All these RFLPs are located between the exons 8 and 9 and lay in an area with unknown function. A different case of RFLP is FokI. This polymorphism was described in the early nineties in the exon 2, and consisted of a C to T change being the truly functional polymorphism of VDR therefore LD analysis is not required for it. The change is inside a start codon (ATG), so when the C variant is present, an alternative start site is used leading to a protein with different size therefore also called Start Codon Polymorphism (SCP). Two protein variants corresponding to the two available start sites exist- a long version of the VDR protein (f allele or M1 form, that is the methionine at first position) and protein shortened by three amino acids (F allele also referred to as the M4 form that is the methionine at fourth position). M4 form was found to be more active than the M1 form in terms of being more transcriptionally potent.

The association of alleles of different polymorphisms in synteny with each other within a population is called linkage disequilibrium (LD). The low level of recombination over time in a certain area of a gene, leads to the presence of certain polymorphisms with a high level of association. In cases of high levels of LD, this leads to blocks of alleles that are present together forming a haplotype. Those blocks vary in size, having an average size of 10 - 20 kb, and they could be very useful to determine the causes of certain genetic diseases. Several studies have been performed to determine the degree of LD among the known polymorphisms of the VDR gene. So far, the information available is very limited but a strong degree of LD has been found among the TaqI, BsmI, EcoRV and ApaI RFLPs with five different haplotypes. Furthermore,

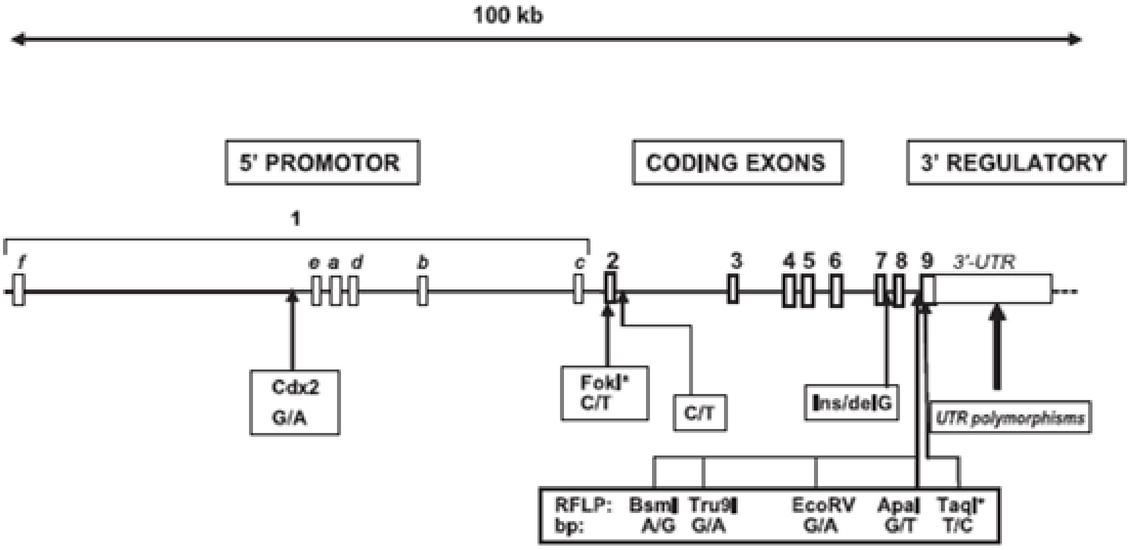


Figure 37: Exon-intron structure of the VDR gene and position of known polymorphisms. Indicates that these polymorphisms are in the coding sequence.
$\star$ *Source* : *GeneReview*, 2004.

Uitterlinden et al. (1996) have shown a strong LD between the BsmI RFLP and a polymorphism present in the 3' UTR (VNTR). Thus, the study of the LD among the different VDR polymorphisms could give new insights in the etiology of certain diseases.

2.3.2.1.1 Activation and effects of Vitamin D on target cells

It has been shown that vitamin D plays an important role in homeostasis and other metabolic pathways, such as those involved in the immune response and cancer (Haussler et.al. 1998). Vitamin D, derived from the diet or by bioactivation of 7-dehydrocholesterol, is inert and must be activated to exert its biological activity. Vitamin D 3 is produced in the skin by an ultraviolet light-induced photolytic conversion of 7-dehydrocholesterol to previtamine D3 followed by thermal isomerization to vitamin D3. The first step in the metabolic activation of vitamin D is hydroxylation of carbon 25. This reaction occurs primarily in the liver, although other tissues including skin, intestine, and kidney have been reported to catalyze 25-hydroxylation of vitamin D. The second and more important step in vitamin D bio activation, the formation of 1,25 (OH)2D3 from 25(OH)D3 occurs, under physiological conditions, mainly in the kidney (Fraser et.al.1970). The renal enzyme responsible for producing 1,25(OH)2D3, 25(OH)D-1a-hydroxylase, is located in the inner mitochondrial membrane and is a cytochrome P-450 monooxygenase requiring molecular oxygen and reduced ferredoxin.

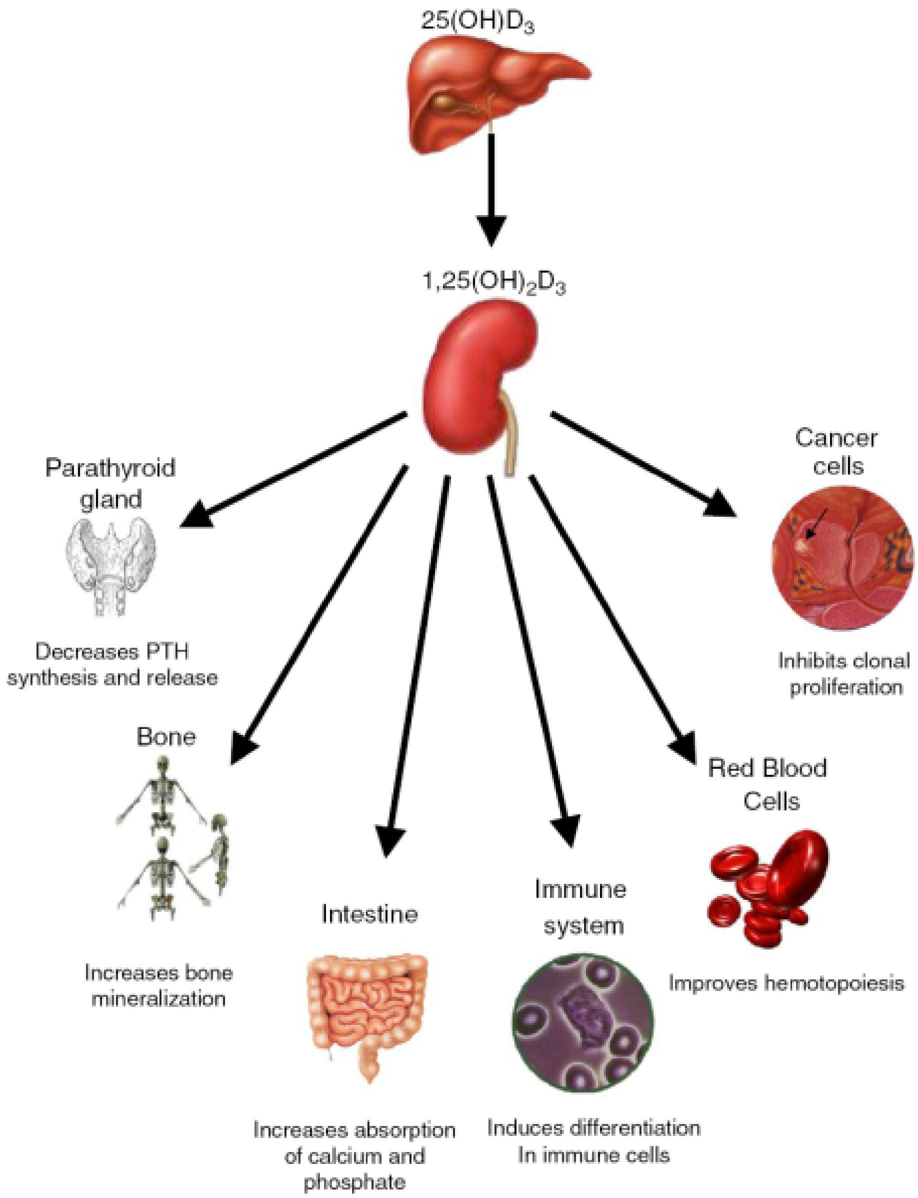


Figure 38: Effects of Vitamin D on target cells
* *Source* : *GeneReview*, 2004.

2.3.2.1.2 VDR gene polymorphism and tuberculosis

Vitamin D induced host defense - Insight into vitamin D-induced antimicrobial activity by human monocytes and macrophages against M. tuberculosis was first suggested by experiments in the laboratories of Rook in 1986 and Crowle in 1987. These experiments were performed adding the active vitamin D3 hormone (1,25D3) to M. tuberculosis infected human monocytes and macrophages in vitro, which resulted in reduction of intracellular bacterial load. These studies opened new questions regarding the role of vitamin D in the physiological response to M. tuberculosis, and the identity of the vitamin D-dependent antimicrobial effectors. There have been many genetic and epidemiological studies linking gene polymorphisms and environmental factors to tuberculosis susceptibility, and a large majority focused on various aspects of the vitamin D pathway. Several studies have linked serum 25D levels to both tuberculosis disease progression and susceptibility. In 1985, a study reported that of 40 Indonesian patients with active tuberculosis and treated with anti-tuberculosis chemotherapy, the 10 patients with the highest 25D levels at the outset of therapy has 'less active pulmonary disease'. Another aspect of the VDR pathway that has been extensively studied is the VDR itself. There are two major VDR polymorphisms that have been studied in terms of tuberculosis susceptibility, known as Taq I and Fok I, with conflicting results. However, the associations became clear in a study examining the relationship between vitamin D deficiency and VDR polymorphisms with tuberculosis in the Gujarati Asians living in west London in the year 2000. The study reported that both the Taq I tt and Fok I ff alleles were associated with tuberculosis only when the individual exhibits serum 25D deficiency. Collectively, these studies have demonstrated that vitamin D plays an important role to host defense against M. tuberculosis in vivo. However, the in vitro studies used the active 1,25D metabolite to stimulate antimicrobial activity, but the association to tuberculosis was with the 25D form. A potential mechanism, reported in 2006 by which the 25D status of an individual may alter their ability to mount an innate immune response against M. tuberculosis. In humans, activation of TLR2/1 results in the induction of key genes in the vitamin D pathway, including the vitamin D receptor (VDR) and the 25-hydroxyvitamin D3-1a-hydroxylase (CYP27b1), which converts the vitamin D prohormone (25D) into the active form (1,25D). Under conditions where levels of the prohormone form of vitamin D (25D) is present at sufficient levels, TLR2/1 activation of monocytes results in a CYP27b1- and VDRdependent expression of the antimicrobial peptide, cathelicidin, and direct microbicidal activity against intracellular M. tuberculosis. Interestingly, the human but not murine cathelicidin promoter contains VDREs, perhaps suggesting a point of divergent evolution between mice and humans in the antimicrobial effectors used by the TLR-mediated innate immune response. Inhibition of the VDR resulted in ablation of the TLR2/1-induced antimicrobial activity, suggesting that VDR activation is a critical step in the innate immune response against M. tuberculosis. Therefore, potentially explaining the association of 25D serum levels to susceptibility to tuberculosis, where low 25D levels cannot provide sufficient substrate for CYP27b1-mediated production of 1,25D to activate the VDR-dependent antimicrobial response.

Exposure to mycobacteria does not always result in the infection whether or not

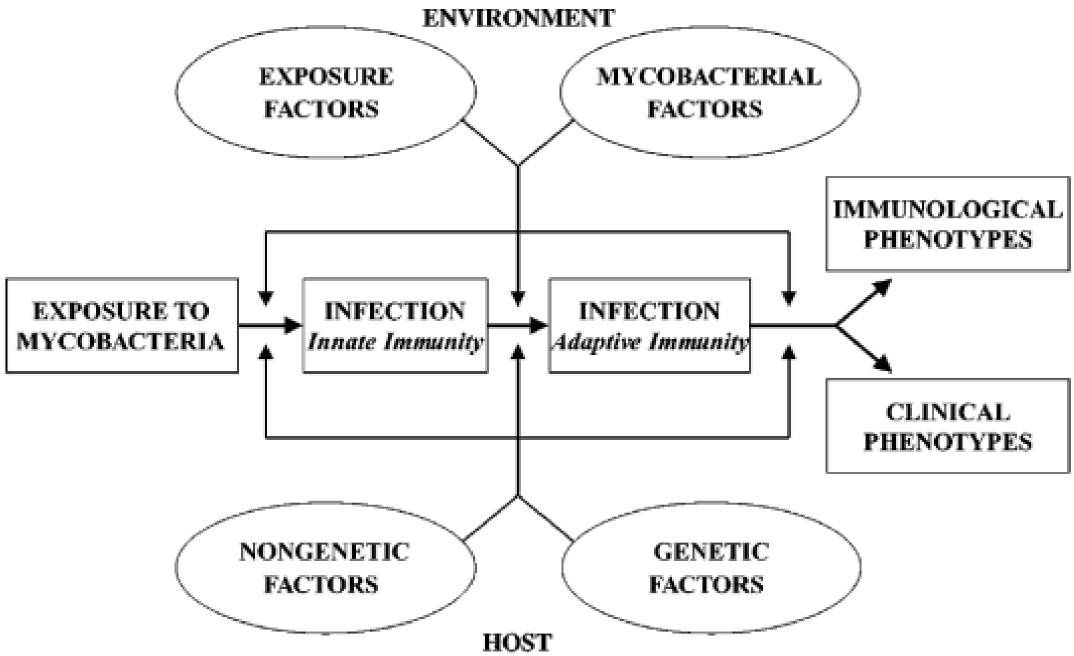


Figure 39: The various steps in the interaction between humans and mycobacteria. † *Source* : *GeneReview*, 2004.

an established infection further develops depends upon innate immunity, alone or in conjugation with adaptive immunity. Immunological and clinical phenotypes may be detected once mycobacterial infection is established and adaptive immunity to mycobacteria is involved. Each of the three steps in this process is under host and environmental control. Host factors may be genetic (e.g., mutation in gene involved in immunity to mycobacteria) or non genetic (e.g., skin lesions) and may have an impact at each satge of the interaction. Environmental factors may be mycobacterial (e.g., virulence factors) or related to the mode of exposure (e.g., direct inoculation) and may have an impact at each stage of the interaction

2.3.2.1.3 VDR polymorphism and renal patients

The effect of VDR polymorphisms in renal failure has been widely explored due to the complex role played by vitamin D in those patients. One of the main complications in patients with chronic renal failure is the development of secondary hyperparathyroidism (sHPT). The complex calcitriol-VDR regulates parathyroid cell proliferation and parathyroid hormone (PTH) synthesis. Thus, the interaction of calcitriol with its receptor inhibits PTH synthesis as well as parathyroid gland cell proliferation. Patients with uremic sHPT were treated with vitamin D metabolites during decades in order to correct the hypercalcemia and, therefore, to inhibit sHPT. The discovery

of the VDR on parathyroid cells opened a new and exciting field of research. Thus, in 1995 Carling et al. (1995) reported a relationship between BsmI polymorphism and primary HPT. The presence of VDR on vascular and cardiac tissue implies new target organs where VDR polymorphisms could be influencing mortality. Furthermore the association of Bsm I genotype with hemoglobin levels and bone mineral loss could explain the increase in survival of some of the patients. It is very difficult to determine the increase in the risk of developing sHPT associated with having a specific BsmI genotype. Firstly, because of the different and, sometimes, contradictory results found in literature. Secondly, due to the fact that the risk calculation is performed with many variables, and some of those change within different studies (sample size, study design, etc.). However, almost all the reports published indicate that there is some influence of BsmI genotype on the sHPT progress. In an ideal scenario in which we would try to prevent sHPT in early stages of CRF, the use of BsmI polymorphism to evaluate the risk of the patients to develop sHPT could be an option.

2.3.2.1.4 VDR polymorphism and bone biology

In 1992, Morrison described for the first time a relationship between the Bsm I polymorphism and osteocalcin levels. The presence of the homozygous allele bb was related to a higher bone mass density (BMD) in normal population and in twin pairs and the presence of the BB genotype was related with lower BMD in postmenopausal women. The sample size used in each study. Overall, what seems to be accepted is that the effect of Bsm I genotype on BMD is relatively small (2-3%) and strongly influenced by some other non-genetic factors like diet. The relationship between bone biology and some other polymorphisms of the VDR gene has also been reported. For instance, the FokI polymorphism has also been associated with differences in BMD but, whereas some papers linked the presence of the longer form of the protein with lower BMD some other reached opposite conclusions. The CDX-2 polymorphism has also been related with BMD in Japanese population, being the allele G associated with lower BMD in lumbar spine. In 1997 two almost simultaneous papers described for the first time the possible relationship between VDR polymorphisms and the risk of suffering osteoarthritis. In one of them, Keen et al. (1997) associated the presence of the T allele of the TaqI polymorphism with a higher risk of suffering osteoarthritis in the knee. Uitterlinden et al. (1997) studied haplotypes of the BsmI, ApaI and TaqI polymorphisms and reached the conclusion that the bAT haplotype was associated with reduced prevalence of knee osteoarthritis. In 1998 Aerssens et al. (1998) demonstrated that BsmI polymorphism had no effect on the prevalence of osteoarthritis in Belgian women with hip replacement.

2.3.2.1.5 VDR polymorphism and cancer

An association has been described between 1,25(OH)2D3 and susceptibility to and outcome of some cancers, like breast, prostate and colon cancers. The relationship includes vitamin D serum levels as well as VDR polymorphisms. In 1997, Ingles et al. (1997) published one of the first reports finding a relationship between the polyA

polymorphism of the VDR gene (one of the UTR polymorphisms) and prostate cancer in the US population. In the meantime, some other reports showing no association between TaqI and/or polyA and prostate cancer were also published. Kibel et al. (1998) and Cheteri et al. (2004) showed no association between both polyA or TaqI polymorphisms and death by prostate cancer in US patients. FokI has been found to be related to prostate cancer in some cases whereas in some others no relationship has been reported. The case of BsmI is similar. Whereas in Japanese and African American populations a relationship has been reported. No influence of the BsmI and risk of prostate cancer has been found in North Americans or Chinese. In addition, in a recent report of Ntais et al. (2003) in which a meta-analysis of 28 different studies was performed, no relationship was found between any of the former VDR polymorphisms and prostate cancer susceptibility. In colon carcinoma, a couple of reports showed association with BsmI polymorphism whereas conflicting results are found regarding FokI. Recently, Halsall et al. (2005) described a new polymorphism in the exon 1 a transcription start site (A-1012G).

2.3.2.1.6 VDR polymorphism and nephrolithiasis

Nephrolithiasis is a multifactorial pathology resulting from the interaction between environmental influences and hormonal and genetic factors. The tendency to form calcium oxalate kidney stones is directly related to urinary concentrations of calcium and oxalate, and inversely to citrate and magnesium. In a paper in 1999, Ruggiero et al. (1999) related the phenotype bb of the Bsm I polymorphism with a higher urinary calcium excretion and thus, to an increase of risk of stone formation. In the same line of reasoning, Mossetti et al. (2003) published a paper showing a decrease of citrate urinary excretion in individuals with the bb phenotype, confirming the theory of a higher risk of kidney stones in the population presenting the b allele. Like in previous cases, these results were not confirmed in all the studies. Nishijima et al. (2002) showed an association of the t allele with a higher calcium excretion and, therefore, to a higher stone formation. Of course, there is also a report demonstrating no association between Taq I polymorphism and stone formation Ozkaya (2003).

2.3.2.1.7 VDR polymorphism and diabetes

The involvement of vitamin D has been suggested in the etiology of both independent and dependent DM. In type 1 DM, Bsm I polymorphism has been linked to susceptibility to present the disease in Southern Indians, Taiwanese, Croatians and Japanese (McDermott 1997). In Finnish and Chilean populations the link could the connection between VDR and diabetes could not be established. Following the same pattern ApaI, TaqI and FokI were found associated to type 2 DM in some reports and not related in some others. In type 2 DM a link between Bsm I and the onset of the disease has been found in Hungarians and Germans but not in French, Bangladeshi or Polish. Similar results have been found regarding the other most common polymorphisms.

2.3.2.1.8 VDR polymorphism and other diseases

The association between the VDR polymorphisms and some other diseases has also been studied. Studies have revealed an association between Bsm I genotype and blood pressure in healthy men, with higher levels of blood pressure in healthy men and women with the b allele Muray et.al. (2003). However, in Korean lead workers, an opposite relationship has been reported Lee (2001). Ortlepp et al. reported an increase in susceptibility to calcific aortic valve stenosis in individuals with the B allele, but also a lack of relationship between the Bsm I polymorphism and the severity of coronary artery disease. However, the same group has recently reported an increase in susceptibility to myocardial infarction associated to the presence of the B allele. These results are in harmony with those of Kammerer et al. (2004) reporting an association of the BB genotype with a higher intimal-medial thickness in carotid artery. Ozaki et al. (2000) and Huang et al. (2002) described a positive relationship between the B allele of the BsmI polymorphism and the incidence of systemic lupus erytematosus in Japanese and Chinese. The same Chinese group provided evidence of a lack of relationship between the Fok I polymorphism and the disease. A link between BsmI and FokI with primary biliary cirrhosis and autoimmune hepatitis has also been found. In multiple sclerosis patients, a higher presence of the bA haplotype has been found . In Caucasian population, Collins et al. (2004) found no association of any of the polymorphisms studied (10 polymorphism, including BsmI and ApaI) with increased susceptibility to suffer Graves' disease. Bellamy et al. (1999) reported that African patients with the tt genotype had some protection against tuberculosis.

2.3.3 Conjecture

Despite the multitude of immune defense mechanisms a host can deploy against M. tuberculosis, the bacteria can continue to persist owing to its repertoire of escape pathways. In this section I shall be linking some of the candidate genes and deriving inference based on the work done on tuberculosis so far.

The recognition and phagocytosis of mycobacteria can occur via mannose receptors or receptors for activation products of the complement system, including complement receptor 1 (CR1). After being phagocytosed, the bacilli are processed into phagolysosomes, and the antigens (Ags) are presented to CD4+ T lymphocytes via major histocompatibility complex (MHC) class II cells. The fusion of phagosomes with endoplasmic reticulum or phagocytosed apoptotic vesicles can favor the presentation of Ags to CD8+ T cells via MHC class I. However, the activation of Toll-like receptors (TLRs) promotes the degradation and release of nuclear factor κ B (NF-κ B) which moves toward the cell nucleus and induces the activation of the transcription of a variety of genes that lead to the production of cytokines such as interleukin (IL)-12 and tumor necrosis factor α (TNF-α), as well as to the expression of co-stimulating molecules such as CD80 and CD86 (which interact with CD28). IL-23, IL-18 and IL-27 are also produced by the macrophages and, together with IL-12, they induce the production of interferon-γ (IFN-γ) by T lymphocytes. The production of IL-2 and IL-2 receptors occurs in activated T cells and induces the proliferation of T lym-

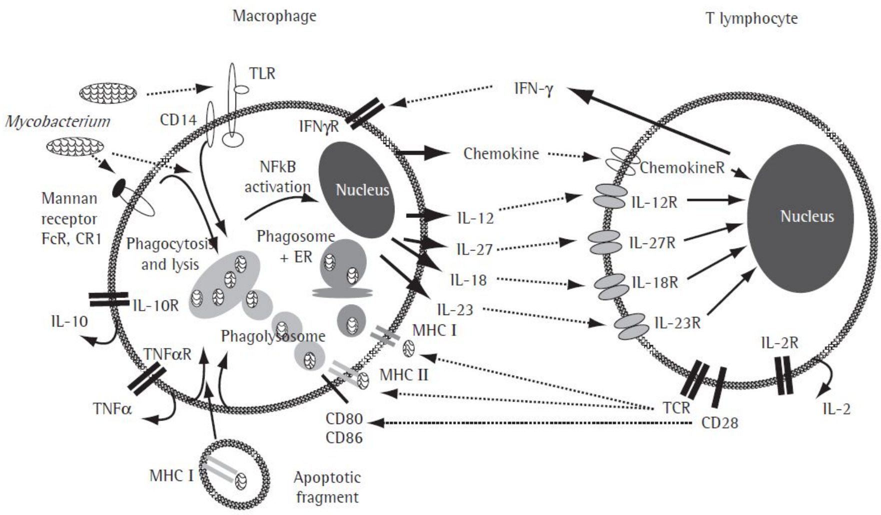


Figure 40: Mechanism involved in the activation of macrophages and T lymphocytes. ‡ $Source$: $Reviewarticle, HenriqueCoutoTeixeiraetal$, 2006.

phocytes. IFN-γ and TNF-α activate macrophage microbicidal mechanisms. IFN-γ is also involved in the production of chemokines. IL-10, produced by macrophages and by T lymphocytes, acts as an endogenous immunosuppressant.

2.3.3.1 Recognition of M. tuberculosis

In order for the innate immune response to successfully defend against pathogens, it must first detect the invader, and then mount the appropriate antimicrobial response. For innate cells, this is accomplished through germ-line encoded pattern recognition receptors, which recognize conserved pathogen associated molecular patterns. Actually Mycobacterium possess specific molecular patterns called pathogen associated molecular patterns (PAMPs) and the host innate immune system recognises these PAMPs by germ line-encoded pattern recognition receptors (PRRs) to elicit immune responses such as the production of proinflammatory cytokines (IFNs) and antigen presentation. M. tuberculosis is known to activate at least two different families of pattern recognition receptors : -

2.3.3.1.1 Tool like receptors

The TLR2 and TLR1 heterodimer recognizes a triacylated lipoprotein derived from M. tuberculosis, which results in activation of NF-κB leading to the production of inflammatory cytokines and direct antimicrobial activity (Smale et al. 2007).

2.3.3.1.2 Nucleotide oligomerization domain like receptors

NOD2 recognizes muramyl dipeptide (MDP), which is a peptidoglycan that can be found on M. tuberculosis (]Yang et al ,2007) Triggering NOD2 similarly leads to a

NF κ B- mediated inflammatory response; however, in contrast to TLRs, NOD2 also results in activation of the inflammasome (Delbridge et al,2007).

2.3.3.2 Macrophage host defense mechanisms against M. tuberculosis

The actions of the inducible nitric oxide synthease (iNOS) and release of nitric oxide (NO) represents a powerful and necessary antimycobacterial defense mechanism in mouse models of tuberculosis infection (MacMicking et al, 1997) Although iNOS has been detected in macrophages from human disease lesions (Nicholson et al,1997), it has not been possible to consistently demonstrate NO-mediated killing in human macrophages. TLR2 activation via bacterial lipopeptide of mouse macrophages induces iNOS promoter activity, production of NO and killing of intracellular M. tuberculosis. However, TLR-induced antimicrobial activity in primary human monocytes and macrophages was not dependent on iNOS activity, nor could NO be detected in these cells. This suggested that human macrophages possess a NO-independent antimicrobial mechanism, but do not exclude a role for NO in human host defense.

2.3.3.3 TLR signalling pathway

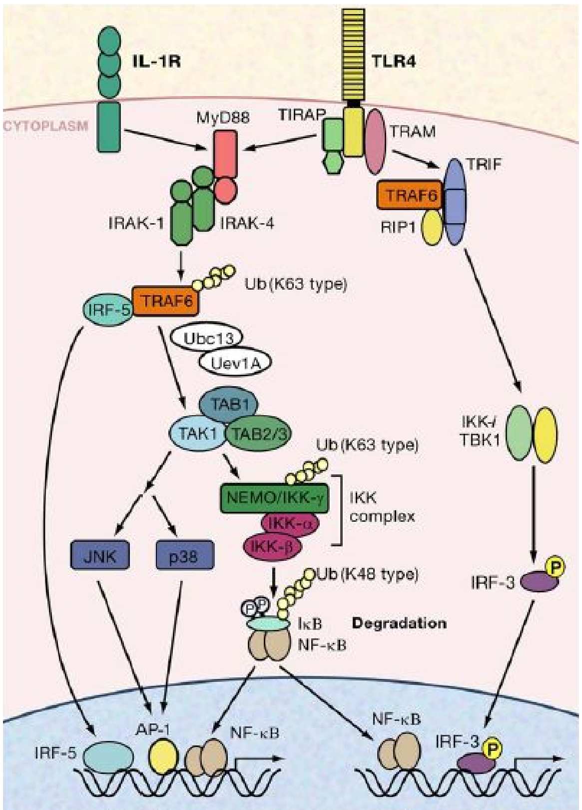


Figure 41: TLR Signaling Pathway TLRs and IL-1R share common signaling pathways in general.

$*$ *Source : Review by Shizuo Akira*, 2006.

Stimulation with their ligands recruits TIR-domain-containing adaptors including MyD88 and TIRAP to the receptor, and the subsequent formation of a complex of IRAKs, TRAF6, and IRF-5 is induced. TRAF6 acts as an E3 ubiquitin ligase and catalyzes the K63-linked polyubiquitin chain on TRAF6 itself and NEMO with E2 ubiquitin ligase complex of UBC13 and UEV1A. This ubiquitination activates the TAK1 complex, resulting in the phosphorylation of NEMO and activation of the IKK complex. Phosphorylated IkB undergoes K48-linked ubiquitination and degradation by the proteasome. Freed NF-κB translocates into the nucleus and initiates the expression of proinflammatory cytokine genes. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. TLR4 triggers the MyD88 independent, TRIF-dependent signaling pathway via TRAM to induce type I IFNs. TRIF activates NF-κB and IRF-3, resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 induce NF-κB activation and TBK1/IKK-i phosphorylate IRF-3, which induces the translocation of IRF-3.

2.3.3.3.1 Autophagy

Autophagy is basically a cellular process by which a cell degrades its own intracellular compartments is a previously unappreciated innate immune defense mechanism. Stimulation of mouse macrophages results with the key immune cytokine, IFN-γ, induces autophagy which is necessary for antimicrobial activity against M. tuberculosis. A separate study demonstrated that lysosomal hydrolyzed ubiquitin peptides have direct antimicrobial activity against M. tuberculosis, and are delivered in an autophagy dependent manner to phagosomes harbouring mycobacterium , representing one possible autophagy-mediated antimicrobial effector. Autophagy can be induced via a variety of methods in macrophages, most immunologically relevant of which includes IFN-γ and activation of TLRs (Gutierrez et al,2004).

Currently, autophagy as an immune mechanism is better characterized in the mouse system, therefore leaving much to be learned about autophagy in the human immune response. There is already strong evidence for autophagy playing a major role in human host defense. Serum starvation and rapamycin induced autophagy-dependent antimicrobial activity against M. tuberculosis in human macrophages is also seen. It remains to be seen if IFN-γ and vitamin D, an important innate immune modulator can also induce autophagy and subsequently antimicrobial activity in human macrophages.

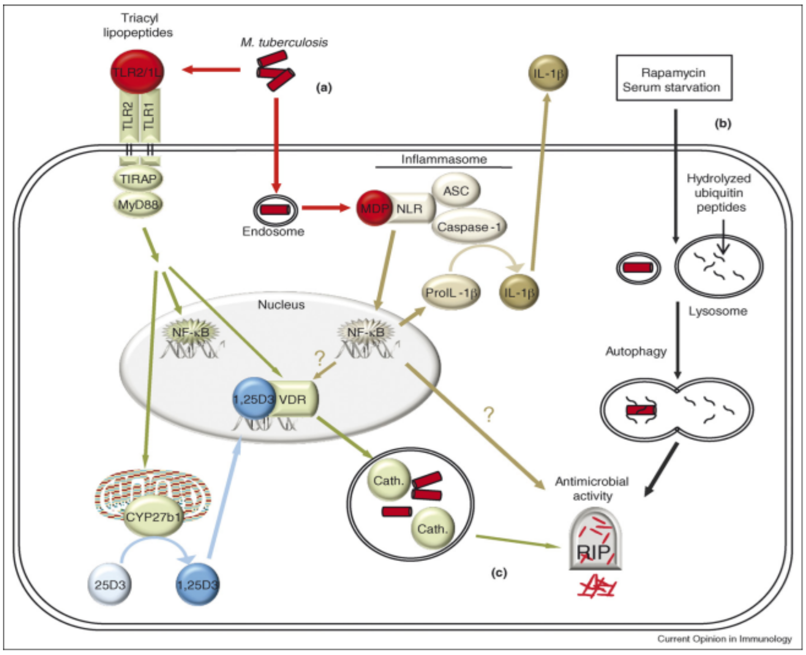


Figure 42: Human macrophage host defense mechanisms against intracellular M. tuberculosis. (a) Recognition of M. tuberculosis is mediated by at least two families of pattern recognition receptors, the Toll-like receptors and the NOD-like receptors. (b) Induction of autophagy results in antimicrobial activity, which delivers hydrolyzed ubiquitin peptides to the lysosome, leading to antimicrobial activity. (c) Although murine macrophages utilize iNOS, human macrophages have evolved to deploy antimicrobial peptides upon TLR-stimulation.

† *Source* : *Current opinion in Immunology*, 2008.

2.3.3.4 TLR and the vitamin D pathway

Scientists have noted a potential mechanism by which the 25D status of an individual may alter their ability to mount an innate immune response against M. tuberculosis. In humans, activation of TLR2/1 results in the induction of key genes in the vitamin D pathway, including the vitamin D receptor (VDR) and the 25-hydroxyvitamin D3-1a-hydroxylase (CYP27b1), which converts the vitamin D prohormone (25D) into the active form (1,25D).

Under conditions where levels of the prohormone form of vitamin D (25D) is present at sufficient levels, TLR2/1 activation of monocytes results in a CYP27b1- and VDRdependent expression of the antimicrobial peptide, cathelicidin, and direct microbicidal activity against intracellular M. tuberculosis.

Interestingly, the human but not murine cathelicidin promoter contains VDREs, perhaps suggesting a point of divergent evolution between mice and humans in the antimicrobial effectors used by the TLR-mediated innate immune response. Inhibition of the VDR resulted in ablation of the TLR2/1-induced antimicrobial activity, suggesting that VDR activation is a critical step in the innate immune response against M.

tuberculosis. Therefore, potentially explaining the association of 25D serum levels to susceptibility to tuberculosis, where low 25D levels cannot provide sufficient substrate for CYP27b1-mediated production of 1,25D to activate the VDR-dependent antimicrobial response. In tuberculosis active Disease the organism is contained within a central core of macrophages surrounded by T cells, that is the granuloma.

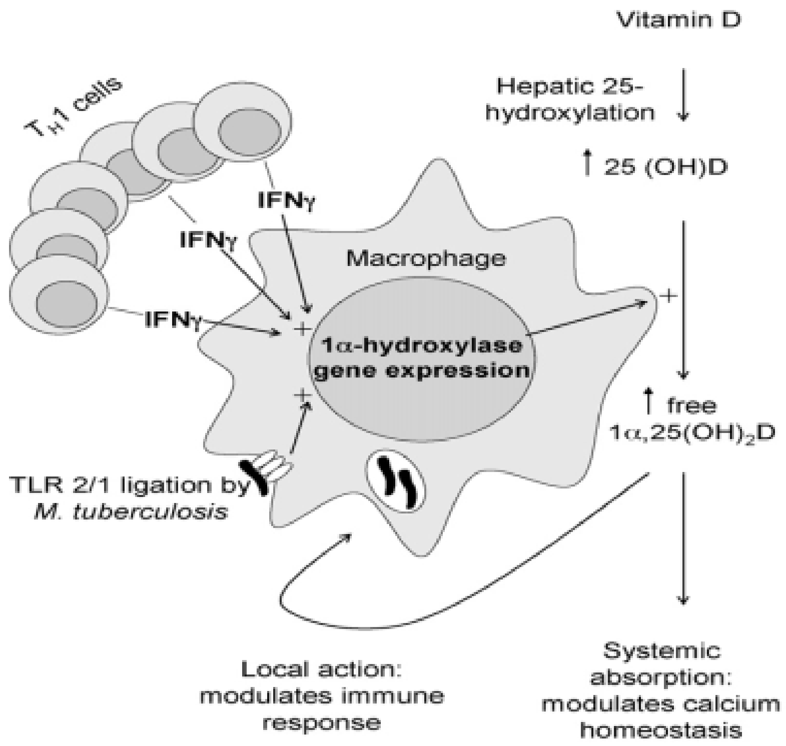


Figure 43: Interconnection between IFNγ, TLR and VDR.
⋆ *Source* : *Journal of steroid biochemistry and Molecular Biology*, 2007.

It is can also be said that expression of macrophage 25-hydroxy-vitamin D 1α-hydroxylase (1α-hydroxylase) is upregulated by ligation of macrophage toll-like receptors by M. tuberculosis antigens. This enzyme metabolises 25-hydroxy-vitamin D (25(OH)D) to 1α,25(OH)2D. Interferon γ (IFNγ) secreted by armed TH1 cells potentiates this effect by upregulating 1α-hydroxylase and inhibiting induction of 25(OH)D 24-hydroxylase , a key enzyme in 1α,25(OH)2D inactivation.

1α,25(OH)2D induces anti-mycobacterial activity in vitro in both monocytes and macrophages . Several mechanisms of action have been described. Exogenous 1α,25(OH)2D induces a superoxide burst (Sly, 2001) and enhances phagolysosome fusion (Hmama,2004) in M. tuberculosis-infected macrophages; both phenomena are mediated by phosphatidylinositol 3-kinase, suggesting that this response is initiated by ligation of membrane vitamin D receptor (VDR) (Norman,2004). 1 α,25(OH)2D also modulates

immune responses by binding nuclear VDR, where it upregulates protective innate host responses (including induction of nitric oxide synthase (Rockett,1998] and cathelicidin (Liu et al 2006) and down-regulates IFN-γ gene expression (by down-regulating activity of the IFNγ promoter).

In addition to this paracrine action, sufficient concentrations of 1α,25(OH)2Dmay be generated to enter the systemic circulation to induce hypercalcemia via action on the kidney, gut and bone. This phenomenon has been observed in patients with active TB who may present with established hypercalcemia or develop hypercalcemia in the early phases of treatment (Sharma et al).

Administration of pharmacologic doses of vitamin D may elevate serum 25(OH)D concentrations to levels at which the ability of vitamin D binding protein (DBP) to bind vitamin D metabolites becomes saturated. A greater proportion of 1α,25(OH)2D synthesised as a result of upregulated macrophage 1α-hydroxylase activity in active TB may therefore be unbound by DBP, resulting in an increase in its biological activity.

Systemically, this has the potential to induce hypercalcemia and ultimately lead to vitamin D toxicity, in which deposition of calcium phosphate crystals in the kidney and vasculature leads to renal failure and hypertension (Pettifor,1995).

The requirement of adequate 25D for the induction of host defence mechanisms via TLR2/1 provided a link between two well documented clinical observations namely black people are

1. More susceptible to virulent tuberculosis infections
2. Have low serum 25D levels.

The biosynthesis pathway of 25D3 in humans involves the absorption of UVB in the skin which converts (7HDC) into a pre-vitamin D3 precursor, and the high melanin content in pigmented skin will absorb UVB rays preventing this reaction [Hagenau et al,2008].

When monocytes cultured in African American sera were stimulated with a TLR2/1 ligand, there was no upregulation of cathelicidin mRNA, whereas monocytes cultured in white sera did. Supplementation of the African American sera with exogenous 25D3 restored the induction of cathelicidin mRNA. This implies that an individual's 25D levels may affect their ability to combat infection, and that supplementation with 25D3 could potentially restore their host defence mechanisms.

2.3.3.4.1 Antimicrobial peptides

Several antimicrobial peptides produced by macrophages have been demonstrated to have direct antimicrobial activity against M. tuberculosis, including cathelicidin (Liu et al,2006), DEFB4 (hBD2) (Rivas et al,2005), and hepcidin (Sow et al,2007). In humans, cathelicidin and DEFB4 were found to contain vitamin D response elements(VDREs) in their promoter regions, but whether or not hepcidin is vita-

min D-regulated is unknown (Wang et al,2004). Activation of the VDR in monocytes/macrophages either directly with 1,25D3 or through TLRs results in the expression of cathelicidin at both the mRNA and protein levels (Martineau et al,2007). siRNA knockdown of 1,25D3 induced cathelicidin in human monocytic cells resulted in complete loss of antimicrobial activity suggesting that antimicrobial peptides represent a major human macrophage host defense mechanism. Furthermore, macrophages can obtain and utilize neutrophil granules, which contain a variety of antimicrobial peptides at high levels, against M. tuberculosis (Silva et al,1989).

2.3.3.5 Interplay between TLR and vitamin D signalling

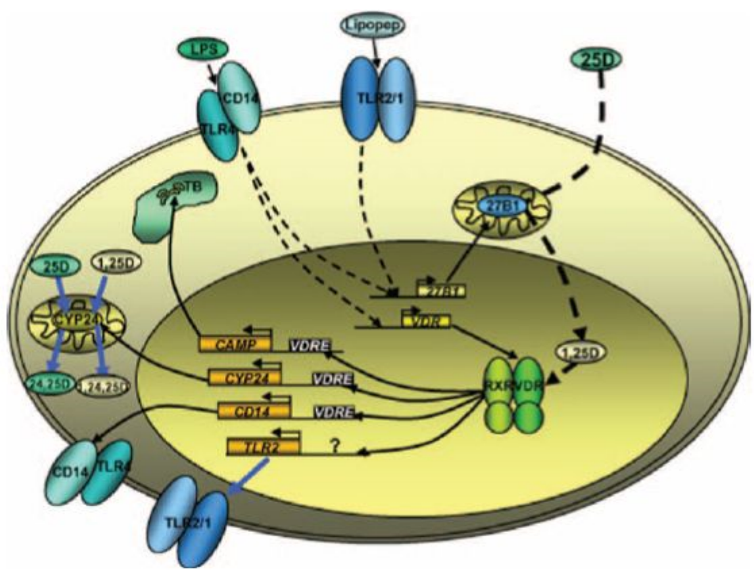


Figure 44: Above mentioned diagram depicts the interplay between TLR and Vitamin D signalling. Blue arrows indicate pathways that are active in keratinocytes but not in monocytes or macrophages.
⋆ *Source* : *Minireview by JohnH. White*, 2008.

Recent studies have found that signalling through human macrophage TLR1/2 heterodimers stimulated with bacterial lipopeptides induced expression of both CYP27B1 and the VDR (Liu et al, 2006). CYP27B1 is known to convert vitamin D prohormone to its active form. Most importantly, they showed that in TLR2/1-stimulated human macrophages cultured in the presence of human serum, downstream VDR-driven responses were strongly dependent on serum 25D concentrations. VDR-driven responses were strongly attenuated or absent in serum from vitamin D-deficient individuals, a defect that could be overcome by 25D supplementation. Moreover, consistent with previous findings (Stead et al, 1990), the 25D levels in serum from African-Americans used in the study were markedly lower than those of Caucasian Americans. This study thus provided a clear demonstration of the dependence of immune responses on circulating 25D levels. Similarly, stimulation of the TLR4/ CD14 receptor complexes

by lipopolysaccharide induces CYP27B1 expression, consistent with correlations that other workers have found between TLR4 and CYP27B1 expression (Evans et al, 2006).

VDR is present in most cells of the immune system, including T lymphocytes, neutrophils, and antigen-presenting cells, such as macrophages and dendritic cells. 1,25D is an inhibitor of maturation of dendritic cells, the most potent of the antigen presenting cells, and acts directly on T lymphocytes to inhibit T-cell proliferation. 1,25D signalling represses the transcription of genes encoding key T helper 1 (Th1) cytokines, such as γ interferon and interleukin. 1,25D is thus a suppressor of antigen presentation to and activation and recruitment of Th1 cells. The net effect of 1,25D action is to polarize T-helper responses toward a more regulatory Th2 phenotype, which is considered a key component of its capacity to suppress Th1-driven autoimmune responses. As Activated macrophages and dendritic cells express CYP27B1, which unlike the renal enzyme, is not regulated by Ca homeostatic signals but is regulated primarily by immune inputs, mainly γ interferon and agonists of the Toll-like receptor (TLR) pattern recognition receptors. Critically, this renders the immune system responsive to circulating levels of 25D which is the prohormone and substrate for CYP27B1. The expression of CYP24, the mitochondrial enzyme that initiates 1,25D catabolism, is extremely sensitive to the presence of 1,25D; the negative feedback loop appears to be defective in macrophages. Ren and colleagues have recently shown that while expression of CYP24 transcripts is induced by 1,25D in macrophages as in other cells, the corresponding enzymatic activity is virtually undetectable. Because 1,25D induces the expression in macrophages of a splice variant form (CYP24-SV) that encodes a truncated enzyme lacking the critical amino-terminal mitochondrial targeting sequence. Although the substrate binding pocket of CYP24-SV is apparently functional, the enzyme, trapped in the cytosol, appears to be catalytically inactive suggesting that, in macrophages, robust 1,25D signalling is maintained over an extended period of time, which would be advantageous for combating intracellular pathogens such as M. tuberculosis. It also provides at least part of the molecular basis for the excessive production of 1,25D by macrophages in granulomatous diseases such as sarcoidosis.

A study has also showed that vitamin D signalling enhanced the expression of TLR2 approximately twofold in human keratinocytes. Given that signalling through either TLR2 or TLR4 enhances vitamin D signalling by upregulating expression of the VDR and CYP27B1, the effects of 1,25D on TLR2 and CD14 expression in keratinocytes constitute a positive feedback loop. Notably, however, recent findings suggest that such a loop does not function in monocytes. Treatment of human monocytes with 1,25D suppressed expression of both TLR2 and TLR4 mRNA and protein in a time- and dose-dependent manner. Signalling through TLR2 was suppressed in 1,25D-treated monocytes, as was signalling through TLR4 in the presence of lipopolysaccharide, even though CD14 expression was induced by 1,25D.

The authors speculated that downregulation of pattern recognition receptors by 1,25D in antigen-presenting cells may contribute to the capacity of 1,25D to attenuate ex-

cessive Th1-driven inflammatory responses and potential downstream autoimmunity. Given that the VDR is a transcription factor and acts as a ligand-regulated gene switch, its signalling is ideally suited to analysis using genomic approaches. Analysis showed that CD14 was induced 27-fold by 1,25D in well-differentiated human squamous carcinoma cells, for example, and in silico analysis identified an upstream VDRE in the human CD14 gene. In the course of in silico screening for VDREs, scientists noted that two genes encoding the antimicrobial peptides (AMPs) cathelicidin antimicrobial peptide (CAMP, hCAP18, LL37) and DEFB2 (DEFB4, β-defensin) contained promoter-proximal consensus DR3-type response elements (Wang et al, 2004). Further analysis of the CAMP and DEFB2 VDREs showed that both elements bound VDR/RXR heterodimers in a 1,25D-dependent manner in vitro and in cells in culture and functioned in reporter gene assays.

CAMP expression was strongly stimulated by 1,25D in all cell types examined (epithelial cells, macrophages/monocytes, and neutrophils), whereas DEFB2 expression was modestly induced in cells of epithelial origin. The strong induction of CAMP by 1,25D was subsequently observed by other workers in a range of cell types, including in 1,25D-treated or UVB-irradiated human skin biopsies, clearly indicating that 1,25D is a primary inducer of the gene. On the other hand, 1,25D is likely to be a secondary regulator of DEFB2 expression. AMPs are vanguards of innate immune responses against bacterial, fungal, and viral attack, and many act directly by disrupting the integrity of pathogen membranes. In addition, CAMP and some β-defensins can function as chemoattractants for neutrophils, monocytes, and other cellular components of immune responses. The induction of AMP expression by 1,25D in humans provides a potential molecular basis for the accumulating evidence, documented above, that a vitamin D-replete state provides broad protection against a range of bacterial and viral pathogens.

2.3.3.6 Cross talk between JAK-STAT, TLR, and ITM dependent pathways in macrophage activation.

As we know that Macrophage phenotype and activation are regulated by cytokines that use the Jak-STAT signaling pathway, microbial recognition receptors that include TLRs, and immunoreceptors that signal via ITAM motifs. The amplitude and qualitative nature of macrophage activation are determined by crosstalk among these signaling pathways. Basal ITAM signaling restrains macrophage responses to TLRs and other activating ligands, whereas strong ITAM signals synergize with the same ligands to activate cells strongly. Similarly, basal ITAM signalling augments IFN signaling and function of receptor activatorof NF-κB, but extensive ITAM activation inhibits Jak-STAT signaling. Thus, intensity and duration of ITAM signaling determine whether ITAM coupled receptors augment or attenuate TLR and Jak-STAT responses.

IFN-γ synergizes with TLRs in part by suppressing TLR-induced feedback inhibition, mediated by IL-10 and Stat3, by a mechanism that depends on glycogen synthase kinase (GSK)3 regulation of AP-1 and CREB. IFN-γ suppresses TLR2 and TLR4

induction/activation of AP-1 by overlapping mechanisms that include regulation of MAPKs, GSK3-dependent suppression of DNA binding, and decreased Fos and Jun protein expression and stability. IFN-γ suppression of TLR-induced activation of AP-1 and downstream target genes challenges current concepts about the inflammatory role of AP-1 proteins in macrophage activation and is consistent with a role for AP-1 in the generation of non inflammatory osteoclasts. Jak-STAT, TLR, and ITAM pathways are basally active in macrophages and strongly induced during innate responses. Thus, signal transduction crosstalk is regulated in a dynamicmanner, which differs under homeostatic and pathologic conditions, and dysregulation of signal transduction crosstalk may contribute to pathogenesis of chronic inflammatory diseases. The Cross talk is depicted in the below mentioned diagrams.

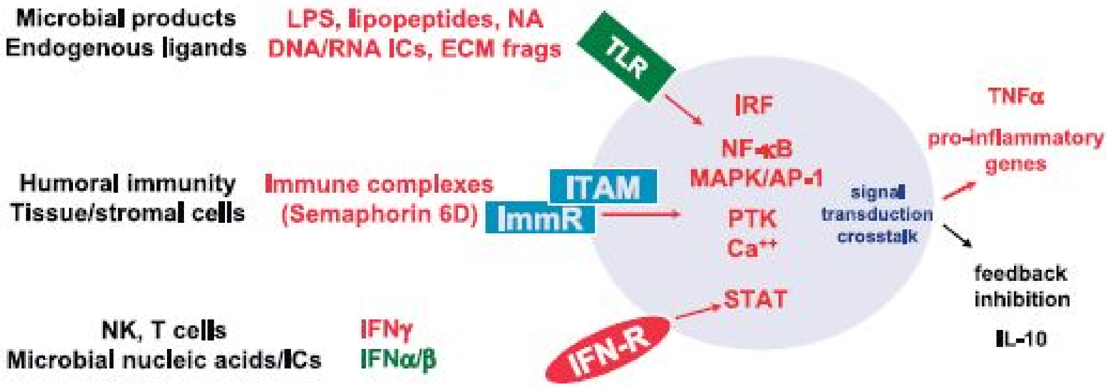


Figure 45: Crosstalk among major macrophage classical activation pathways.
$*$ *Source* : *Journal of Leukocyte Biology*, 2007.

TLR signaling results in the downstream activation of three major families of proteins important in activating inflammatorygene expression: NF-κB/Rel proteins, IFN regulatory factors (IRFs), and MAPKs. ITAM signaling, similar to TLR signaling, activates NF-κB and MAPKs and thus can modulate TLR-induced activation of these molecules and also, more strongly activates tyrosine kinase cascades and calcium signaling. IFN-γ is one of the most potent endogenous macrophage-activating factors and uses the Jak-STAT signaling pathway. Semaphorin 6D- ITAM-containing receptor interactions are indirect and mediated by plexins.

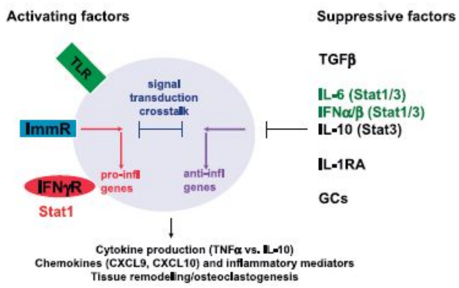


Figure 46: Balance of cytokine action determines severity of inflammation. Many suppressive factors are induced as part of homeostatic feedback inhibition. Crosstalk at the signal transduction level contributes to cytokine balance.

$\star$*Journal of Leukocyte Biology*, 2007.

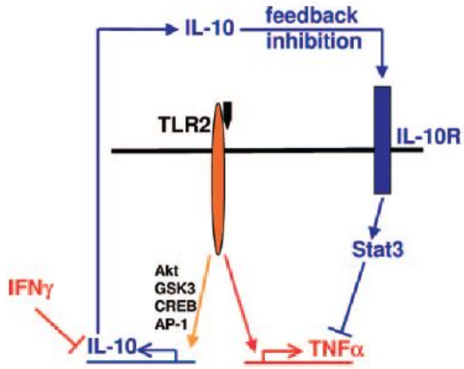


Figure 47: IFN-γ suppresses TLR2-induced feedback inhibition by decreasing IL-10 production and downstream Stat3 activation, thereby interrupting a TLR2-induced feedback-inhibition loop. Suppression of IL-10 expression results in increased production of the inflammatory cytokines such as TNF-γ.

† *Source* : *Journal of Leukocyte Biology*, 2007.

IFN-γ suppresses TLR2-induced Akt activity, linked with decreased phosphorylation and increased activity of GSK3β, which in turn, contributes to inhibition of AP-1 DNA binding. Diminished AP-1 and CREB activity in IFN-γ-primed, TLR2-activated cells leads to decreased expression of AP-1 and CREB target genes such as IL-10. In IFN-γ primed macrophages, constitutively active GSK3 could also potentiate activation of NF-κB and promote the expression of proinflammatory mediators such as TNF-α.

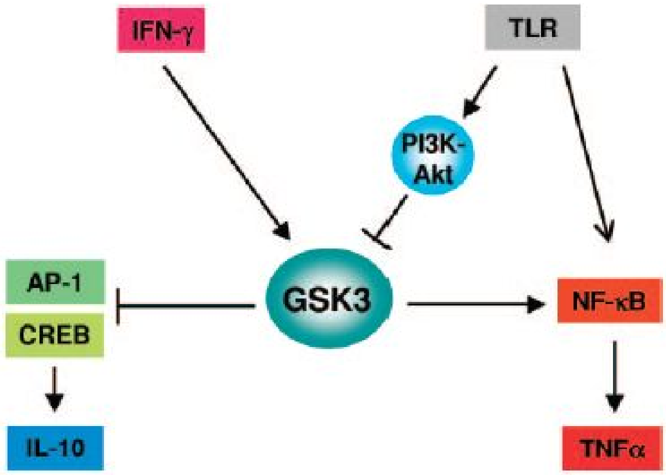


Figure 48: GSK3 integrates inflammatory signals and regulates the balance of cytokine production.
‡ *Source* : *Journal of Leukocyte Biology*, 2007.

2.3.3.7 Connection with NOS2

The L-arginine-nitric oxide (NO) and vitamin D pathways comprise two parallel systems utilized by macrophages to kill MTB, NO generated from the amino acid L-arginine has a clearly established role in MTB killing by murine macrophages. Although early reports cast doubt on the importance of the L-arginine-NO pathway in human macrophage killing of MTB. Within the past 2 years, evidence has emerged in humans for an immunomodulatory role of L-arginine via an NO-independent pathway, namely via modulation of T-cell function through L-arginine's effect on the expression of CD3, a component of the T-cell receptor (TCR) (Zea et al, 2006).

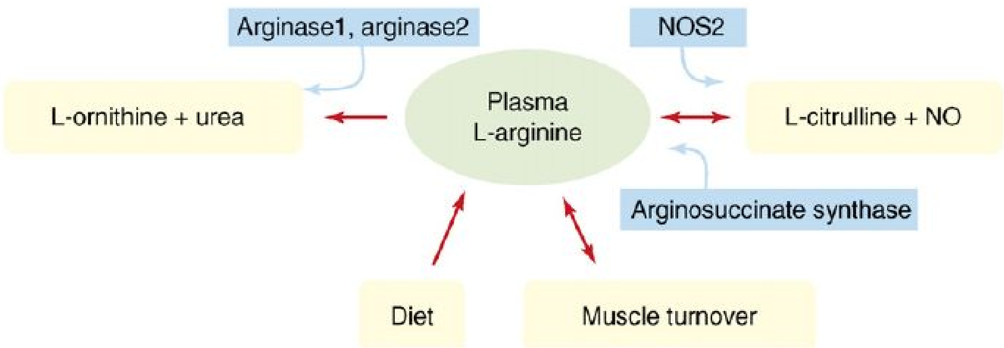


Figure 49: Metabolic fate of L-arginine and macrophage nitric oxide production.
∗ *Source* : *Review by Anna P. Ralph, Cell Press*, 2008.

Sources of L-arginine (the active biological enantiomer of this conditionally essen-

tial amino acid) in the fed state include exogenous (food) and endogenous sources (whole-body protein degradation and, to a small extent, de novo synthesis from citrulline by renal arginosuccinate synthase). L-arginine is converted to NO by NOS2, the inducible product of the NOS2 gene. Th1 cytokines (principally IFN-γ) stimulate expression of macrophage NOS2 leading to NO production, whereas under the influence of Th2 cytokines (IL-4, IL-10 and IL-13), arginine is depleted by arginases. NO is said to possess antimicrobial activity.

Study Type	Results
L-arginine-derived nitric oxide and other RNIs	
In vitro MTB culture	MTB isolates are sensitive to NO and other RNI and differ in their RNI-susceptibility.
Mouse macrophage studies.	Arginine-derived RNI in mouse macrophages effectively kill MTB. MTB lacking genetic resistance to RNI cannot grow in mouse macrophages, in contrast with wild-type MTB.
Human macrophage studies	Alveolar macrophages from healthy humans infected *ex vivo* with MTB produce NO, and NO production correlates with intracellular growth inhibition of MTB. Blood mononuclear cells from healthy donors infected *ex vivo* with MTB and from people with pre-existing TB infection produce NO. Pulmonary macrophages kill mycobacteria only if they express NOS2; killing is prevented with a NOS inhibitor.
In vivo mouse studies	NOS2 is expressed at sites of disease in immunocompetent mice. Fulminant MTB disease develops in *NOS2* knockout mice (*NOS2*$^{-/-}$) and in mice with a variety of immune defects, in contrast with controls. MTB infection is poorly contained in mice treated with NOS inhibitors.
In vivo human studies	In lung resection studies NOS2 and nitrotyrosine (a tissue marker of NO metabolism) are expressed in macrophages within granulomata and areas of TB pneumonitis. NOS2 expression is increased in peripheral blood monocytes from people with TB compared with healthy controls. NOS2 is expressed in macrophages from lungs of patients with tuberculosis. Pulmonary NO production measured by eNO is significantly higher in TB patients than in controls; eNO is inversely associated with disease severity and decreases with anti-TB treatment.
Relationship between CD3ζ expression and L-arginine in TB and other conditions	
Human T cells *in vitro*	Depletion of L-arginine *in vitro* causes reduced CD3ζ expression, impaired T-cell signaling and diminished proliferation. Arginase-mediated L-arginine depletion induces downregulation of CD3ζ; addition of L-arginine *ex vivo* leads to CD3ζ re-expression and recovery of T-cell proliferation.
In vivo human TB study	Peripheral blood T cells from patients with pulmonary TB have decreased CD3ζ expression, correlating with arginase-mediated L-arginine deficiency, which normalises with successful TB treatment.

Table 9: Immunological evidence for the role of arginine and NO in TB control.
† *Source* : *Review by Anna P. Ralph, Cell Press*, 2008.

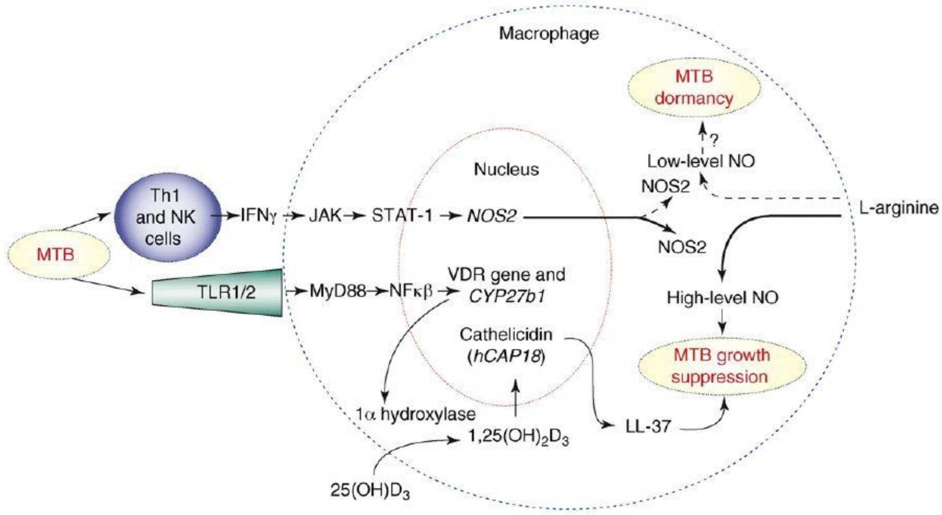


Figure 50: Scheme of macrophage antimycobacterial pathways.
† *Source* : *Review by Anna P. Ralph, Cell Press*, 2008.

Parallel pathways [IFN-γ JAK and STAT- L-arginine-derived NO] and [TLR2-MyD88 and NFκB- vitamin D-cathelicidin and LL37] lead to macrophage activation and mycobacterial control. IFN-γ release by Th1 and NK cells (stimulated by MTB-infected antigenpresenting cells) engages the JAK and STAT-1 pathways within the macrophage, leading to upregulation of genes including NOS 2. Interaction of MTB with TLR in monocytes and macrophages (mainly via TLR1/2) leads to engagement with MyD88 and NF-κB. The subsequent cascade of antimycobacterial responses includes TNFα production, IL-12 release (recruiting Th1- mediated immunity, providing another means of IFN-γ production and further macrophage activation) and upregulation of the vitamin D3- cathelicidin pathway. High NO concentrations (e.g. resulting from high L-arginine concentration and NOS 2 expression) are associated with MTB growth inhibition, whereas low-level NO (e.g. resulting from L- arginine or NOS2 insufficiency) appears to be associated with MTB dormancy.

2.3.3.7.1 Interaction between NO, and VDR pathways

Macrophages can be activated either by TLR triggering, leading to increased intracellular vitamin D3 and cathelicidin induction, or by IFN-γ, acting via Janus tyrosine kinase (JAK) and signal transducer and activator of the transcription (STAT-1)-signalling pathway to upregulate NOS2 and thereby increase NO release. Interactions between the L arginine-NO and vitamin D-TLR-cathelicidin pathways are probable. First, CYP27b1 requires an extracellular source of L-arginine for full expression and is upregulated by NO in an avian macrophage cell line. Second, the antimycobacterial effect of vitamin D might be mediated in part by NO. Third, nuclear factor κ B (NF- κB), a downstream mediator of TLR signalling, also might impact on NOS2 regulation and NO production in murine macrophages. These in vitro studies cannot determine how these immunological strategies manifest in the infinitely complex in

vivo setting of human TB infection in which there are individual variations in vitamin D and L-arginine concentrations both systemically and locally in the vicinity of the granuloma. Comparative clinical trials of both arginine and/or vitamin D as adjunctive therapies in TB could assess the relative importance of these two immune pathways in the human host.

Part III

Aims, and objectives

3 Aims, and objectives

DNA sequence variations which occur frequently in the population are called polymorphisms. Their abundance in the human genome as well as their high frequencies in the human population have made them targets to explain the variation in the risk as well as resistance to Tuberculosis as well as other infectious diseases. Keeping the above points in mind, the study was designed and it was proposed to : -

1. Study the allele distribution of polymorphic $(CA)_n$ microsatellite repeat of IFNγ and Fok I polymorphism of VDR gene in patients with tuberculosis from Chhattisgarh and Orissa Population.

2. Compare the frequency of different alleles of $(CA)_n$ microsatellite repeat of IFNγ and Fok I polymorphism of VDR between cases and properly chosen controls.

Part IV

Materials, and Methods

4 Materials

4.1 Methodology for FOK-I polymorphism of VDR

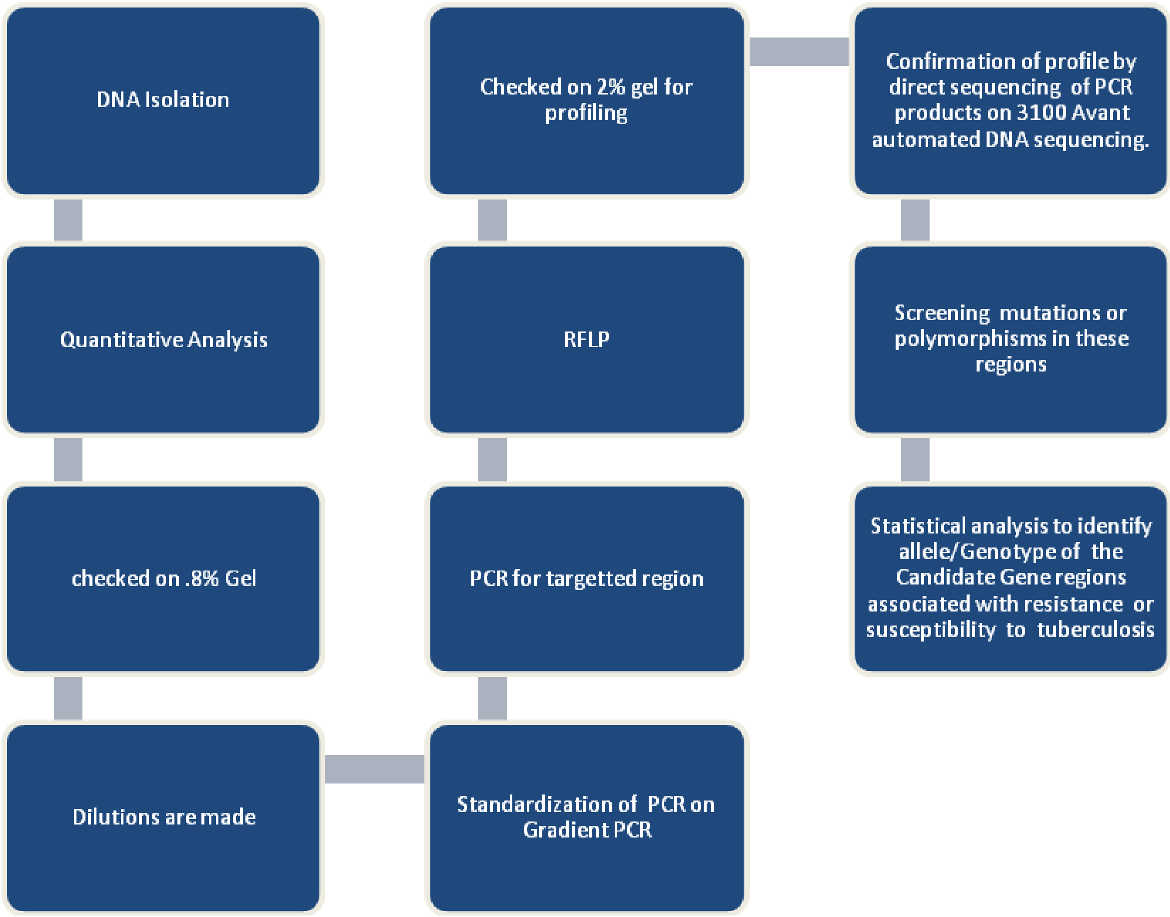


Table 10: Methodology for FOK I polymorphism of VDR.

4.2 Methodology for IFNγ

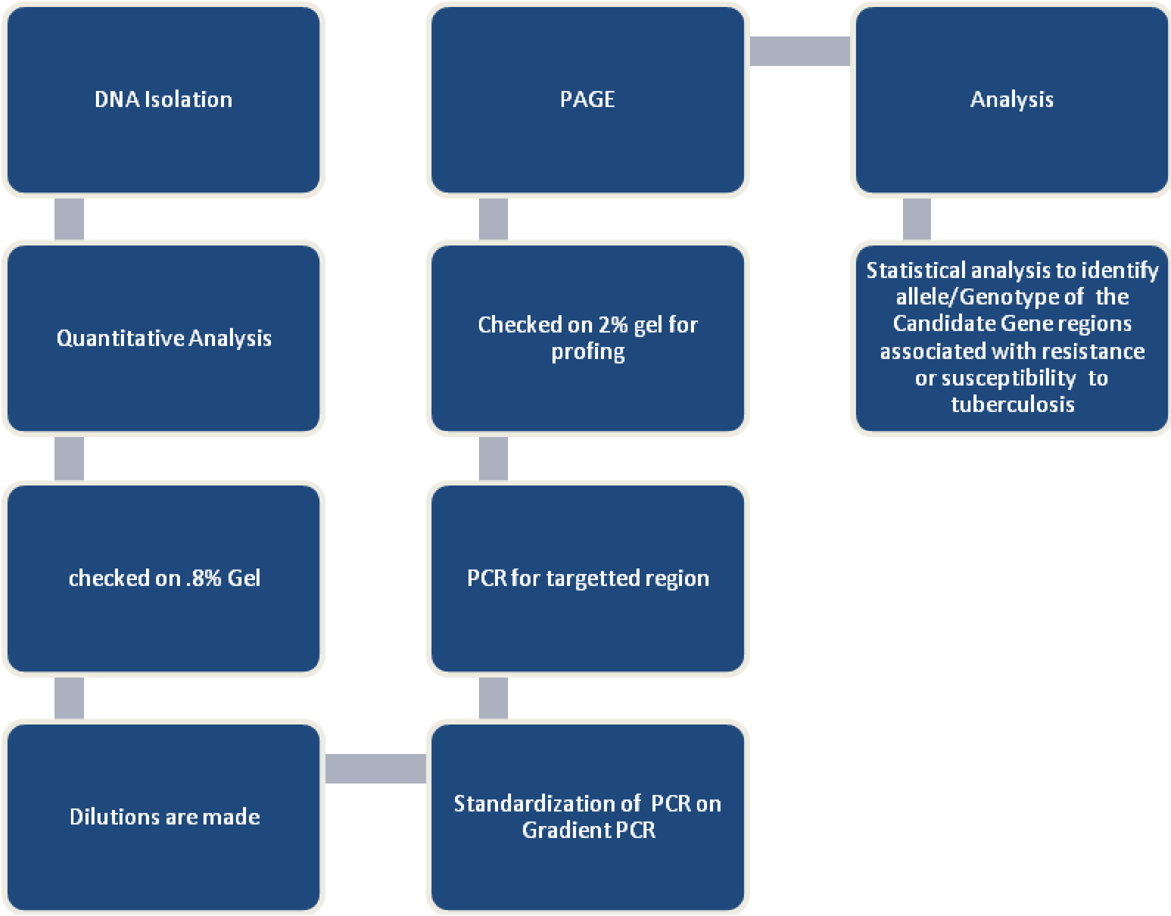


Table 11: Methodology for IFN γ.

4.3 Materials

Acetic acid (qualigens), Acryl amide (sigma), Agar-agar (qualigens), Agarose powder (pronadisa), Ammonium per sulphate (sigma), Boric acid (qualigens), Bromophenol blue (sigma), Chloroform (qualigens), DNA molecular markers (pBR 322 Hae III digested) (promega), Ethanol (Merck and Bengal chemicals.), Ethylene diamine tetra acetate (EDTA) (Merck), Ethidium bromide (sigma), Formaldehyde (qualigens), Hydrochloric acid (SD Fine), 8-hydroxyquinoline(glaxo), Isoamylalcohol (qualigens), β Mercapto ethanol (qualigens), N,N Methylen bisacrylamide (sigma), Magnesium Chloride (qualigens), N, N, N', N' Tetramethylethylene diamine (sigma), Phenol (Merck), Sodium dodosyl sulphate (sigma), Sodium Acetate (qualigens), Silver nitrate (qualigens), sodium hydroxide (qualigens), Sucrose (qualigens), Tris base (sigma), Triton X-100 (New Jersey lab), Xylene cyanol (sigma).

4.3.1 Enzymes

- Taq polymerase (Bangalore Genei)
- Restriction enzyme- FOK I (New England Biolabs)
- NEB Buffer- 4 (New England Biolabs)
- Shrimp Alkaline Phosphatase and Exonuclease-I enzymes (New England Biolabs)
- Big Dye terminator v1.1 and 3.1 cycle sequencing kit (Applied Biosystems)

4.3.2 Instruments

Centrifuge: Heraeus Biofuge primoR, Heraeus Sepatech Biofuge 22R, Megatech centrifuge 450D.
Vortex: REMI CYCLOMIXER.
NanoDrop: ND 1000 spectrophotometer.
UV-Visible Spectrophotometer: LABOMED INC Spectro UV-VIS Double Beam PC scanning.
Electrophoresis apparatus: BIORAD-V20-CDC, BIORAD-HU10, BIORAD-Mini-PROTEIN® II cell.
Power pack: BIORAD computer controlled model 3000xi, CONSORT E-802.
PCR Machine: Gene Amp® PCR system 9700 (Applied Biosystems), Programmable thermal controller-100™ (MJ RESEARCH, INC.).
UV Transilluminator: SPECTROLINE® ultraviolet transilluminator.
ABI PRISM® 3130 DNA Analyser: The ABI PRISM® 3130 DNA Sequencer automatically analyses DNA molecules labelled with multiple fluorescent dyes. It consists of a charge couple device (CCD) camera and a power macintosh computer that includes software for data collection and data analysis. After samples are loaded onto the system's vertical gel, they undergo capillary electrophoresis, laser detection, and computer analysis. electrophoretic separation can be viewed on-screen in real-time.

4.3.3 Cases and controls

A total of 96 individuals from Chhattisgarh and Orissa were studied for the germ line status of polymorphisms in the CA microsatellite repeat in the promoter region of IFNγ gene and single nucleotide polymorphisms in VDR gene. This included 48 patients with tuberculosis and 48 unrelated healthy controls with no history of tuberculosis form the same cohort. Care was taken that both cases and controls were ethnically and geographically matched. Needed ethical concerns were taken in consideration for the sample collection and study. DNA was extracted from peripheral blood samples of the individuals using the standard phenol-chloroform method.

4.3.4 Reagents for DNA isolation

The following solutions were prepared, sterilized in different experiments : -
Lysis I buffer (10X): 155mM $NH4Cl_2$, 10mM K2CO3, 1mM EDTA.
Lysis II buffer (1X): 8ml of 5M NaCl, 1ml of 1M Tris, 400μl of 0.5M EDTA.
10% SDS solution.
Tris Saturated Phenol: Phenol, 0.1% 8-Hydroxy Quinoline, 0.5 M Tris HCl (pH8.0), 0.1 M Tris HCl (pH 8.0).
Chloroform: Isoamylalcohol (24:1): 24 ml of Chloroform was added to 1 ml Isoamylalcohol.
100% Ethanol: 100 % absolute alcohol.
70 % Ethanol: 70 ml of absolute alcohol in 30 ml DDW.
TE Buffer (100ml): 10mM Tris HCl (pH 7.5), 1mM EDTA (pH8.0).

4.3.5 Reagents for agarose gel electrophoresis

Agarose powder.
10X TBE Buffer: 108 gm Tris HCl, 54.8 gm boric acid, 9.28 gm EDTA dissolved in 1000 ml autoclaved water, use after autoclaving.
Ethidium Bromide: 10 mg/ml (stock).

4.3.6 Reagents for PCR

Reagents for Polymerase Chain Reaction (PCR):
50ng/μl DNA sample: as target.
10 X PCR Buffer: 15mM MgCl2 [ABI]
10 X PCR Buffer: without MgCl2 [ABI]
2.5mM dNTPs (dATP, dTTP, dGTP, dCTP each) [ABI]
Primers: 12.5 Pico-moles/μl [Sigma Aldrich]
Taq DNA Polymerase: 3U/ μl [Sigma Aldrich]

4.3.7 Primer designing with oligo, and primer3

Primer Designing using OLIGO and primer 3:
The primers for amplification of the desired region were designed using OLIGO software. Certain points that were kept in mind during primer designing are : -
Primers should be nearly 18-25 nucleotides in length. The GC content of the primers should be between 40% to 60%. The Tm values of the members of a primer pair should not differ by greater than 3°C. The 3‘ end of each primer should be G or C if possible. The primers should have no inverted repeats or self-complementary sequences.
The primer set designed for amplification of the desired promoter region from the VDR gene is as follows.
VDR = F5'AGCTGGCCCTGGCACTGACTCTGCTCT3'

VDR = R5'ATGGAAACACCTTGCTTCTTCTCCCTC3'

4.3.8 Reagents for polyacrylamide gel electrophoresis

Acryl amide: N, N'- Methylene bis acryl amide (19:1): 19gm acryl amide and 1gm N, N' - Methylene bis acryl amide dissolved in 50 ml autoclaved water and made final volume up to 100 ml, covered the bottle with foil and filter the solution in dark using whatmann filter paper. 10% Ammonium persulphate: dissolve 0.1 gm of Ammoniumpersulphate in 1 ml autoclaved water. TEMED - N, N, N', N' -Tetra methyl ethylenediamine solution 10X TBE buffer- Tris, Boric Acid and EDTA Reagents used for Silver Staining: 10 %(V/V) Ethanol 0.1 gm% AgNO3: 0.1 gm AgNO3 powder dissolved in 100 ml autoclaved water. 1.5gm% NaOH: 1.5 gm of NaOH powder dissolved in 100 ml of distilled water. Formaldehyde: 400μl/100ml of 1.5%NaOH.

4.3.9 Reagents for silver staining

10 %(V/V) Ethanol 0.1 gm% AgNO3: 0.1 gm AgNO3 powder dissolved in 100 ml autoclaved water. 1.5gm% NaOH: 1.5 gm of NaOH powder dissolved in 100 ml of distilled water. Formaldehyde: 400 μl/100ml of 1.5%NaOH.

4.4 Methods

Methodology

4.4.1 DNA isolation

1. Add 1.5ml of Lysis I in 0.5ml of blood. Vortex to mix properly and store at -200°C for 5-10 minutes.
2. Centrifuge at 5000rpm for 8 minutes.
3. Discard the supernatant and beat the pellet by vortexing vigorously.
4. Repeat the first three steps to obtain clear pellet.
5. Beat the pellet again.
6. To the pellet add 500 μl of Lysis II. Vortex to mix properly.
7. Add 80 μl of 10%SDS and vortex again.
8. Incubate overnight at 370C (or at 550C for 15-30 minutes).
9. To the mixture add 150 μl of phenol and 100 μl of chloroform, mix gently.
10. Centrifuge at 6000rpm for 5 minutes.

11. Collect the aqueous supernatant and discard the pellet.
12. Add 50 μl of chloroform to the supernatant and mix gently.
13. Centrifuge at 8000rpm for 5 minutes.
14. Collect the supernatant.
15. Add chilled absolute ethanol to the supernatant and spool the DNA.
16. Wash the DNA with 70% ethanol by centrifuging at 12000rpm for 2 minutes.
17. Dry the pellet and dissolve it in sterilized T.E. buffer.

4.4.1.1 Brief explanation

In order to extract DNA from the cell, we have to purify it by separating it from other cellular components as the quality of starting material remains a crucial important factor for most purposes. Although there are many different methods for purifying nucleic acids based on the starting material, they have a number of basic features in common. The starting material could be a culture of bacterial or Eukaryotic cells, which would simply need to be separated from the growth medium (by centrifugation), or a more complex tissue sample, which needs to be homogenized so that individual cells can be lysed. Wherever possible, the material should be freshly harvested or frozen until ready to use, to avoid degradation by enzymes present in the cell extract. The cells then need to be lysed to release their components, the nature of the treatment will vary widely according to the cell type. Bacterial cells have walls that have to be broken before the cell contents can be released. This is usually accomplished by using lysozyme (An enzyme naturally present in egg white and tears for the very purpose of breaking down bacterial cell walls), often in conjuction with EDTA and detergent such as SDS (sodium dodecyl sulphate). EDTA eliminates divalent cations and thus this stabilizes the outer membrane in bacteria such as E. coli, and also inhibits DNAses that would otherwise tend to degrade the DNA, while the detergent will solubilise the membrane lipids.Plant and fungal cells have cell walls that are different from bacteria and require alternative treatment either mechanical or enzymatic, while animal cells (which lack a cell wall) can usually be lysed by more gentle treatment with a mild detergent. After breaking up cell walls and plasma membrane, mixture of that material and intracellular components are left. Now a complex solution of DNA-RNA-proteins-lipids and carbohydrates are released.A sudden lysis of the cell will usually result in some fragmentation of chromosomal DNA. In particular the bacterial chromosome, which is usually circular in its native state will be broken in linear fragments. Where it is necessary to obtain very large (even intact) chromosomal DNA, gentler lysis conditions are necessary. Bacterial plasmids, however are readily obtained in their native, circular state by standard lysis conditions. The next step is to separate the desired nucleic acid from these other components.

4.4.1.1.1 Enzyme treatment

Removal of RNA from DNA preparation is easily achieved by treatment with ribonuclease (RNAase). Since RNAase is a very heat stable enzyme, it is easy to ensure that it is free from traces of deoxyribonuclease (DNAase) that would otherwise degrade DNA, simply by heating the enzyme before use. Removal of DNA from RNA preparations used to be less easy since it requires DNAase without any RNAase activity. However, it is now possible to buy RNAase free DNAase (as well as DNAase free ribonuclease). Protein contemplation can be removed by digestion with a proteolytic enzyme such as proteinase K. These treatments are applied if necessary in different nucleic acid purification protocols. However, in some protocols they are omitted either because the contemplation is unimportant for a specific purpose or because the contaminats will be removed any way through subsequent steps as described.

4.4.1.1.2 Phenol chloroform extraction

Removal of protein is particularly very important as the cell contains a number of enzymes that will degrade nucleic acids, as well as other proteins that will interfere with subsequent procedures by binding to the nucleic acids. A classical and still very frequently employed way of removing proteins is by way of extraction with liquefied phenol, or preferably a mixture of phenol and chloroform. Phenol and chloroform are (largely) immiscible with water, and so we get two layers (phases) when added to cell extract. When the mixture is vigorously agitated, the protein will be denatured and precipitated at the interface. If we are using phenol that has been equilibrated with neutral or alkaline buffer (as normally the case) the nucleic acid (DNA and RNA) will remain in the aqueous layer. On the other hand if we carry out the extraction with acidic phenol DNA will partition into the organic phase allowing us to recover RNA from the aqueous phase. Phenol is naturally acidic, so equilibration with water or the use of an acidic buffer will produce the appropriate conditions. Phenol extraction is

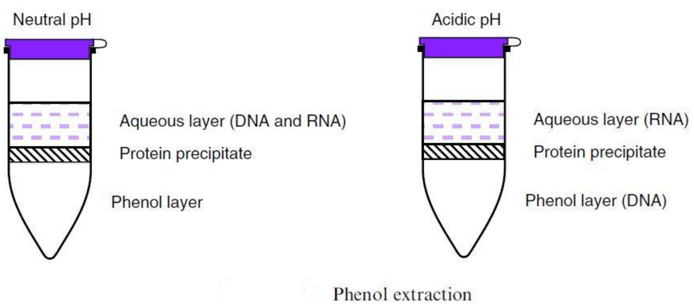


Figure 51: Phenol Extraction.

also useful in subsequent stages of manipulation when it is necessary to ensure that all traces of enzymes have been removed before proceeding to the next step. Phenol is highly toxic by skin absorption and gloves must be worn.

4.4.1.1.3 Alcohol precipitation

Following phenol extraction, we have a protein free sample of nucleic acids. However, it will probably will be more dilute than required, and furthermore, it will contain traces of phenol and chloroform. Phenol in particular does have a significant degree of solubility in water and could lead to denaturation of enzyme in later steps. The answer is normally to concentrate and further to purify, the solution by precipitating the nucleic acids. This is done by adding an alcohol, either isopropanol or (more frequently) ethanol in the presence of monovalent cations (like sodium, potassium, ammonium) a nucleic acid precipitate forms which can be collected at the bottom of the test tube by centrifugation. Some of the salt will precipitate as well and is removed by washing with 70% ethanol. The details of alcohol precipitation procedures vary

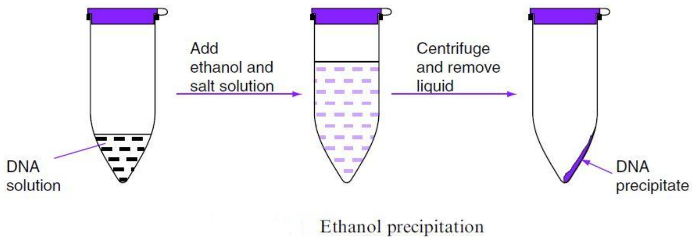


Figure 52: Ethanol precipitation.

according to the nature of the nucleic acid; for example, low molecular weight DNA is much less readily precipitated. This is not a major factor in the purification of DNA from cell extracts, but does need to be considered at later stages, such as when purifying small fragments of DNA.

4.4.2 Gradient centrifugation

Centrifugation at moderate speeds is frequently used in DNA purification for the separation of particulate matter from a solution, whether it be the removal of cellular debris or the recovery of precipitated nucleic acids. It is also frequently employed in various column purification methods.

4.4.3 Quantitation of DNA

The DNA was quantified using the dual beam UV spectrophotometer at wavelength 260 nm and 280nm. The amount of DNA was calculated using following formula.
DNA μg/ml =OD at 260 nm x Dilution factor x 50 μg/ml
Ratio at 260/280 was taken as criteria to check the purity of DNA. The ratio should be 1.8, which indicated purity of DNA. Ratio < 1.8 indicates protein contamination and >1.8 indicates RNA contamination. Running an aliquot of DNA sample in 0.8% agarose gel containing Ethidium bromide also checked the purity of DNA.

4.4.3.1 Nanodrop

Nanodrop was initiated by applying 1μl of water. 1μl of TE buffer was applied on the column as a blank. Readings of samples were taken in DNA 50 program.

4.4.3.2 Brief explanation

Nanodrop works on the principle of Beer Lambert's Law. The absorption spectrum is characteristic for a particular element or compund, and does not change with varying concentration. It is based on the absorption of quanta of light by a chemical substance due to the promotion of electrons from one atomic orbital to another in that substance. The wavelength of incident photon will determine the energy level of excitation according to Plank's law. While the relative intensity of the several absorption lines does not vary, at a given wavelength the measured absorbance has been shown to be proportional to the molar concentration of the absorbing species and the thickness of the sample the light passes through. This is known as Beer-Lambert Law. The plot of absorption versus wavelength for a particular compound is measured using the nanodrop, which disperses the transmitted light using a diffraction grating and subtracts from the known incident spectrum to determine opacity at each wavelength in the measured range.

4.4.3.3 Beer Lambert's Law

When radiation fall on homogeneous medium, a portion of light is reflected is reflected, a portion is absorbed and the remainder is transmitted. The two laws governing the absorption of radiation are known as Lambert's law and Beer's law. In combined form they are referred to as Beer-Lambert law.
Lambert's Law:
It states that when monochromatic light passes through a transparent medium, intensity of transmitted light deceases exponentially as the thickness of absorbing material increases. Beer's Law:
It states that the intensity of transmitted monochromatic light decreases exponentially as the concentration of the absorbing substance increases. A=εbc Where A is absorbance (no units, since A = log10 P0 / P), e is the molar absorbtivity or extinction coefficient with units of L mol$^{-}1cm^{-}1$, b is the path length of the sample - i.e. the path length of the cuvette in which the sample is contained (in cm) and finally, c is the concentration of the compound in solution, expressed in mol L^{-}1. We can also express transmittance in terms of concentration, but the Beer-Lambert law is more useful because the relationship between absorbance and concentration is linear. Beer's law is linear in most cases, except:

- At high concentrations
- If there is scattering of light due to particulates in the sample
- If the sample fluoresces or phosphoresces
- If the radiation is not monochromatic

- If there is stray light

High concentration results in non-linearity because at high concentration, we have strong electrostatic interactions between molecules, we may get changes in refractive index and if we have a system in chemical equilibrium, equilibrium may shift at high concentrations hence is graph is deviated toward the negative or positive side.

4.4.4 Agarose gel electrophoresis

Agarose gel mixture (0.8 to 2.0% agarose, 1X TBE and 3-4 μl Ethidium bromide) was poured in gel casting tray fitted with a comb. It was allowed to polymerize for approximately 30 minutes at room temperature. The comb was removed after polymerisation. The gel was put into running buffer and 2 μl DNA samples with loading dye were loaded in each well. The PCR samples were run at 120 Volt and genomic DNA samples were run at 80 Volt for 10-20 minutes. After run, the gel was visualized in UV illumination.

4.4.4.1 Brief explanation

Electrophoresis is defined as the movement of ions and charged macromolecules through a medium when an electric current is applied. Agarose and polyacrylamide are the primary stabilizing media used in the electrophoresis of macromolecules. Macromolecules are separated through the matrix based on size, charge distribution and structure. In general, nucleic acids migrate through a gel based on size, with little influence from base composition or sequence, whereas proteins separate through the matrix based on size, structure and charge because their charge density is not proportional to size. Two equations are relevant to the use of power supplies for electrophoresis of macromolecules: Ohm's law and the second law of electrophoresis. These two laws and the interactions of these parameters (watts, volts, current) are critical to understanding electrophoresis. Ohm's law states that: I = V/ R Where, I = current, V = voltage, and R = resistance. This tells us that the current is directly proportional to the voltage, and inversely proportional to the resistance. Resistance of the system is determined by the buffers used, the types and configurations of the gels being run, and the total volume of all the gels being run. The second law states that: $W = I \times V$
Where, W = power, I = current, and V = voltage
It states that power or watts is equal to the product of the current and voltage. Since $V = I \times R$, this can also be written as $W = I^2 \times R$. During electrophoresis, one of the parameters is held constant and the other two are allowed to vary as the resistance of the electrophoretic system changes. In vertical systems, the resistance of the gel increases as highly conductive ions such as Cl^- are electrophoresed out of the gel.
As these ions are removed from the gel, the current is carried by less conductive ions such as glycine, borate, acetate, etc. Under normal conditions in horizontal systems, there is little change in resistance. However, with high voltage or extended runs in horizontal systems, resistance can decrease.

4.4.5 Constant electrophoresis parameters

There are advantages and disadvantages for setting each of the critical parameters as the limiting factor in electrophoresis. Sequencing gels are often run at constant wattage to maintain a uniform temperature. Agarose and acrylamide gels for proteins and DNA resolution are generally run at constant voltage. The relevant issues are as follows : Constant wattage In the vertical system when the wattage is held constant, the velocity of the samples will decrease because the current, which is in part carried by the DNA, decreases to compensate for the increase in voltage. The generation of heat will remain uniform, resulting in a constant gel temperature. F the current should decrease disproportionately (from a buffer problem, buffer leak or a hardware problem), the power supply will increase the voltage to compensate. Since voltage and current vary over time at a constant wattage, it is not possible to predict the mobility of samples from the calculation of watt-hours.

4.4.5.1 Constant current

When the current is held constant, the samples migrate at a constant rate. Voltage and wattage will increase as the resistance increases, resulting in an increase in heat generation during the run. If a break occurs in the system, such as a damaged lead or electrode or a buffer leak, the resistance of the gel will be greatly increased. This will cause a large increase in wattage and voltage resulting in generation of excessive heat. It is possible even for the system to get hot enough to boil, or scorch or burn the apparatus.

4.4.5.2 Constant voltage

When the voltage is set constant, current and wattage will decrease as the resistance increases, resulting in a decrease of heat and DNA migration. Since the heat generated will decrease, the margin of safety will increase over the length of the run. If a problem develops and the resistance increases dramatically, the current and wattage will fall since the voltage cannot increase. Even if the apparatus fails, the worst that is likely to happen s that the resistance will increase so much that the power supply will not be able to compensate and it will shut off.

4.4.6 Physical chemistry of agarose

Agarose is a natural polysaccharide isolated from agar, which is obtained from various species of marine algae. Agarose has become an important material in life science research and related areas because of its distinctive physical and chemical properties as a gel matrix.

4.4.6.1 Agarose as a gel matrix

Agarose is derived from a series of naturally occurring compounds present in seaweed. Most agar comes from various species of Gelidium and Gracilaria. All species contain ester sulphates and some, except Gracilaria, contain varying amounts of pyruvates.

Gracilaria agarose contains methyl esters, the position of which is variable according to the species. Agarose consists of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-a-L-galactopyranose. This basic agarobiose repeat unit forms long chains with an average molecular mass of 120 kDa, representing about 400 agarobiose units. There are also charged groups present on the polysaccharide, most notably pyruvates and sulphates.

4.4.6.2 Advantages of Agarose as a gel matrix

Agarose forms a macroporous matrix which allows rapid diffusion of high molecular weight (1000 kDa range) macromolecules without significant restriction by the gel. Agarose gels have high gel strength, allowing the use of concentrations of 1% or less, while retaining sieving and anticonvective properties. Agarose is non-toxic and, unlike polyacrylamide, contains no potentially damaging polymerization byproducts. There is no free radical polymerization involved in agarose gelation. Rapid staining and destaining can be performed with minimal background. Agarose gels are thermoreversible. Low gelling temperature and low melting temperature agaroses permit easy recovery of samples, including sensitive heat-liable materials. Agarose gels may be air dried.

4.4.6.3 Properties of agarose

The charged groups present on the polysaccharide- pyruvates and sulphates- are responsible for many of the properties of agarose.

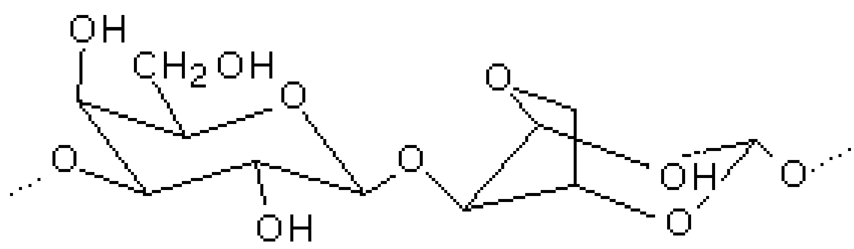


Figure 53: Structure of agarose.

4.4.6.4 Gelation

The mechanism for the gelation of agarose was first suggested by Rees and later demonstrated by Arnott et. al. it involves a shift from a random coil in solution to a double helix in the initial stages of gelation, and then to bundles of double helices in the final stage. The average pore size varies with concentration and type of agarose, but is typically 100-300 nm.

4.4.6.5 Gel strength

One of the most important factors contributing to the success of agarose as an anti-convection medium is its ability to exhibit high gel strength at low concentration (<6%). Gel strength is defined as the force, expressed in g/cm^2, which must be applied to fracture an agarose gel of a standard concentration. The gel strength of a specific lot of agarose decreases over time because of spontaneous hydrolysis of the agarose polysaccharide chains. This loss of gel strength can be particularly noticeable after 5 years from the manufacture date.

4.4.7 Agarose buffer system

The most commonly used technique for DNA separation is submerged horizontal electrophoresis in 0.5 - 6.0 % agarose, usually in TAE and TBE buffer. By varying the agarose concentration and/or buffer, it is possible to separate reliably double - stranded DNA in the size range of approximately 20 - 50,000 base pairs.

4.4.7.1 The choice between TAE, and TBE

If the DNA is less than 12- 15 kb and will not be recovered from the gel, then either TAE or TBE buffer at a standard concentration can be used. For larger DNA, the best choice is TAE in combination with low field strength. During these extended electrophoretic runs, the larger gel porosity and low field strength reduce the tendency of the DNA to smear. TBE buffer is preferred for separation of small DNA molecules (<1kb) when recovery is not required because the interaction between TBE and agarose results in a smaller apparent pore size. This tighter gel reduces the broadening of DNA bands owing to dispersion and diffusion. Whichever buffer is used, the depth over the gel in a horizontal electrophoretic system should be 3-5 mm, with fewer buffers the gel may dry out during electrophoresis. Excessive buffer depth decreases DNA mobility, promotes band distortion and can cause excessive heating within the system.

4.4.8 Loading buffers

They increase the density of the sample, ensuring that the DNA settles evenly into the well. They add colour to the sample and so simplify loading. They contain mobility dyes which migrate in electric field towards the anode at predictable rates: this enables the electrophoretic process to be monitored.A number of loading buffers are commonly used for agarose gel electrophoresis. They are usually as 6X or 10X stock solutions. Alkaline loading buffer is used when performing alkaline gel electrophoresis.The inclusion of the lower molecular weight glycerol in the loading buffer does not always prevent the DNA from streaming up the sides of the well before electrophoresis has begun can result in a U-shaped band.In TBE gels, glycerol also interacts with borate which can alter the local pH. A loading buffer with too hgh an ionic strength causes bands to be fuzzy. Ideally, the DNA sample should be resuspended in the same solution as the running buffer. If this is not possible, use a sample buffer with a lower ionic strength than the running buffer. The zone of highest resolution for

DNA separation in an agarose gel is in the middle third of the gel. The voltage gradient is determined from the distance between the electrodes, not the gel length. If the voltage is too high, band streaking, especially for DNA =12-15 kb, may result. When the voltage is too low, the mobility of small DNA =1 kb, is reduced and band broadening occurs owing to dispersion and diffusion.

4.4.8.1 Detection of DNA in agarose gels

Ethidium bromide is a fluorescent dye which detects both single and double stranded DNA. However, the affinity for single stranded is relatively low compared with that for double stranded DNA. Ethidium bromide contains a planer group which intercalates between the bases of DNA, resulting in an increase in fluorescence yield when the DNA/ Ethidium bromide complex is exposed to UV radiations. At 254 nm, UV is absorbed by the DNA and transmitted to the dye; at 302 nm and 366 nm, UV is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum.For optimal resolution, sharpest bands and lowest background, the gel is stained following electrophoresis. Ethidium bromide is added to the agarose solution while it is cooling at a final concentration of 0.1 to 0.5 μg/ml (with stock solution of 10 mg/ml). Ethidium bromide can also be included in the gel and electrophoresis buffer (0.5 mg/ml) with only a minor loss of resolution. The electrophoretic mobility of DNA will be reduced by approximately 15%. DNA in agarose gels can also be stained by other substances like silver staining, methylene blue and acridine orange. Equipment and Supplies necessary for conducting Agarose Gel Electrophoresis are relatively simple and includes: An electrophoresis chamber and power supply. Gel casting trays, which are available in variety of sizes an composed of UV - transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis. Sample combs, around which molten agarose is poured to form sample well in the gel. Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE) Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to 'fall' into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded. Ethidium bromide, a fluorescent dye used for staining nucleic acids. Trans illuminator (an ultraviolet light box), which is used to visualize ethidium bromide-stained DNA is gels.

4.4.8.2 Casting a gel

To cast a gel, agarose powder was mixed with electrophoresis buffer to the desired concentration and then heated it until completely dissolved. Ethidium bromide was added to the gel (5μ1) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution, it was poured into a casting tray, which was sealed with tape at both ends, containing a sample comb and allowed to solidify at room temperature. (Generally 1% gel is prepared for DNA samples and 2% gel is prepared for RNA. For DNA, TBE buffer is used and for RNA, TAE is used). After the gel had solidified, the comb was removed, using care not to rip the bottom of

the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer on a Para film were then pipetted into the sample well carefully, the lid and power leads were placed on the apparatus, and a current was applied. The voltage applied depends upon the size of the DNA that is to be visualized. Current is confirmed by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode as DNA itself is negatively charged because of the presence of phosphate groups.

4.4.9 Insilico PCR

In-silico PCR searches a sequence database with a pair of PCR primers, using an indexing strategy for fast performance. We used the in-silico PCR tool of UCSC genome browser database (http://genome.ucsc.edu/cgi-bin/hgPcr), to explore the probable amplification by the designed primers and find about the specificity of the primer set under ideal conditions.

4.4.10 Polymerase chain reaction

Our specific region of DNA was amplified by PCR. The essential components for PCR are target DNA sequence, oligonucleotide primers that are complementary to the regions on opposite strands that flank the target DNA, the four dNTPs, thermostable Taq DNA polymerase and buffer to stabilize the enzyme. The basic PCR reaction what we use is as follows : -
Reagents: Total reaction volume 12.5μl for each sample.

Component	**Stock**	**Required**	**Volume**	**Primers and their respective PCR conditions for amplification**
Target DNA	25 ng/μl	50 ng	2μl	FOK I of VDR
Buffer A with $MgCl_2$ (10X)	1.5mM	1mM $MgCl_2$	0.83μl	F5'AGCTGGCCCTGG-CACTGACTCTGCTCT3' R5'ATGGAAACACCT-TGCTTCTTCTCCCTC3'
Buffer B without $MgCl_2$ (10X)			0.42μl	PCR condition 94°C 4 min, 94°C 30 sec, 58°C 30 sec, 72°C 1min, [35 cycles], 72°C 2 min, 4°C 5 min
Primer (Forward)	12.5pg/μl	6.25 pg/μl	0.5μl	CA repeats in IFN γ
Primer	12.5 pg/μl	6.25 pg/μl	0.5μl	F5'CAGACATTCACAAT-TGATTTTATTC3'
Reverse	12.5 pg/μl	6.25 pg/μl	0.5μl	R5'CTGTGCCTTCCTG-TAGGGTA3'
dNTPs	0.025 mM	.2 mM	1μl	PCR condition 94°C 5 min, 94°C 1min, 60°C 1min, 72°C 1min, [32 cycles], 72°C 1 min, 4°C 5 min
Taq polymerase	3 units/μl	.3 units	0.1μl	
PCR water			7.15μl	

Procedure

1. Aliquoted 2 μl of sample DNA in the well of PCR plate.
2. Master mix prepared which constitute PCR water, Buffer A, Buffer B, dNTPs , forward primer, reverse primer and Taq polymerase.
3. Master mix and DNA mixed properly and centrifuged,fixed the program in the Thermocycler
4. After the completion of reaction, PCR amplification is checked by agarose gel (2%) electrophoresis.

4.4.10.1 Brief explanation

Polymerase chain reaction (PCR) allows specific DNA sequences to be copies or amplified over a million fold in a simple enzyme reaction. DNA, corresponding to genes or fragments of genes, can be amplified from samples of chromosomal DNA containing

thousands of genes. Amplified DNA is used for the analysis or manipulation of genes. DNA is amplified by PCR in an enzyme reaction which undergoes multiple incubations at different temperatures. PCR has four key components: Template DNA:
This contains the DNA sequence to be amplified. The template DNA is usually a complex mixture of many different sequences, as is found in chromosomal DNA, but any DNA molecule that contains the target sequence can be used. RNA can also be used for PCR by first making a DNA copy using the enzyme reverse transcriptase.There should be enough copies of template DNA to obtain a signal after 25 to 35 cycles. Preferably = 104 copies, but less than 1 μg of human genomic DNA per 50 μl reaction.If the target DNA concentration is low, more than 35 cycles may be required to produce sufficient product for analysis. DNA polymerase: A number of DNA polymerases are used for PCR. All are thermostable and can withstand the high temperatures (upto 100°C) required. The most commonly used enzyme is Taq DNA polymerase from Thermus aquaticus, a bacterium present in hot springs. The role of the DNA polymerase in PCR is to copy DNA molecules. The enzyme binds to single-stranded DNA and synthesizes a new strand complementary to the original strand. DNA polymerases require a short region of double-stranded DNA to get started. In PCR, this is provided by the oligonucleotide primers, which create short double-stranded regions by binding on either side of the DNA sequence to be amplified. In this way the primers direct the DNA polymerase to copy only the target DNA sequence. Deoxynucleotidetriphosphates (dNTPs):
These molecules correspond to the four bases present in DNA (adenine, guanine, thymine and cytosine) and are substrates for the DNA polymerase. Each PCR required four dNTPs (dATP, dTTP, dCTP,dGTP), which are used by the DNA polymerase as building blocks to synthesize new DNA. Primers:
These are specific short single-stranded DNA molecules of 20 to 21 oligomers obtained by chemical synthesis. Primer sequences may be designed using software package (gene tool) as required. To avoid potential problems, the primers should be purified by gel electrophoresis or HPLC ion-exchange chromatography. Primer sequences should not complement with themselves or to each other, particularly the 3' ends. This avoids template-independent amplification of primer sequences or primer dimer, which can lead to other, larger primer artifacts. Primer-dimer may occur to some extent even without an apparent overlap. Primer concentrations should be optimized very carefully. Low primer concentration will result in little or no PCR product, while too high primer concentration may result in amplification of non-target sequences. Working of a PCR:
PCR allows the amplification of target DNA sequences through repeated cycles of DNA synthesis. Each molecule of target DNA synthesized acts as a template for the synthesis of new target molecules in the next cycle. As a result, the amount of target DNA increases with each cycle unit it becomes the dominant DNA molecule in the reaction. During the early cycles, DNA synthesis increases exponentially but in later cycles, as the amount of target DNA to be copies increases and the reaction components are used up, the increase becomes linear and then reaches a plateau. Each cycle of DNA synthesis involves three stages (denaturation, primer annealing, elongation),

which take place at different temperatures and together result in the synthesis of target DNA. Denaturation:
The reaction is heated to greater than 90°C. At this temperature the double helix is destabilized and the DNA molecules separate into single strands capable of being copies by the DNA polymerase. Primer annealing
The reaction is cooled to temperature that allows binding of the primers to the single-stranded DNA without permitting the double helix to reform between the template strands. This process is called annealing. The temperature used varies (typically 4 - 60°C) and is determined by the sequence and the number of bases in the primers. Extension
The stage is carried out at the temperature at which the DNA polymerase is most active. For Taq, this is 72°C. The DNA polymerase, directed by the position of the primers, copies the intervening target sequence using the single - stranded DNA as a template. A total of 20-40 PCR cycles is carried out depending on the abundance of the target sequence in the template DNA. Sequences up to several thousand base pairs can be amplified. To deal with the large number of separate incubations needed, the PCR is carried out using a microprocessor-controlled heating block known as a thermal cycler. In the first cycle, DNA molecules are synthesized which extend beyond the target sequence. This is because there is nothing to prevent the DNA polymerase continuing to copy the template beyond the end of the target sequence. However in subsequent cycles, newly synthesized DNA molecules, which end with the primer sequence act as templates and limit synthesis to the target sequence so that the amplified DNA contains only the target sequence. Reagents for PCR Reaction:
Primer: Primers are short oligonucleotide sequences used to initiate chain elongation/extension after they got annealed at a proper complementary site. These sites are called primer binding sites. The primers may be specific-used to amplify certain know specific sequences or random - can anneal at any site randomly and amplifies it. The primer always binds at 5' position of a single template strand. 10 x Assay Buffer:
The standard assay buffer for PCR reaction contains 10mM Tris HCI (pH=9), 1.5 mM $MgCl_2$, 50 mM KCI and 0.01% gelatin. The presence of Mg++, a divalent cation is very critical and the concentration of Mg++ is maintained by the assay buffer during PCR reaction. MgCl2: The Mg++ ions of the MgCl2 acts like a chelating agent and also enhances the activities of Taq and dNTPs by catalyzing the reaction. The Mg++ catalyses the polymerization reaction therefore its absence form the reaction mix disrupts the whole reaction. It is available along with the 10 x assay buffer or may be available separately. Too much or too little MgCl2 reduces amplification efficiency or results in amplification of non-target sequences. The MgCl2 concentration should be adjusted in parallel to significant changes in the concentration of sample DNA or dNTPs. This will keep the free magnesium ion constant. For example, reducing the concentration of dNTPs from 200 VmuM each to 40 μM each should be accompanied by a reduction in MgCl2 concentration of 640 μM. Polyacrylamide Gel Electrophoresis:

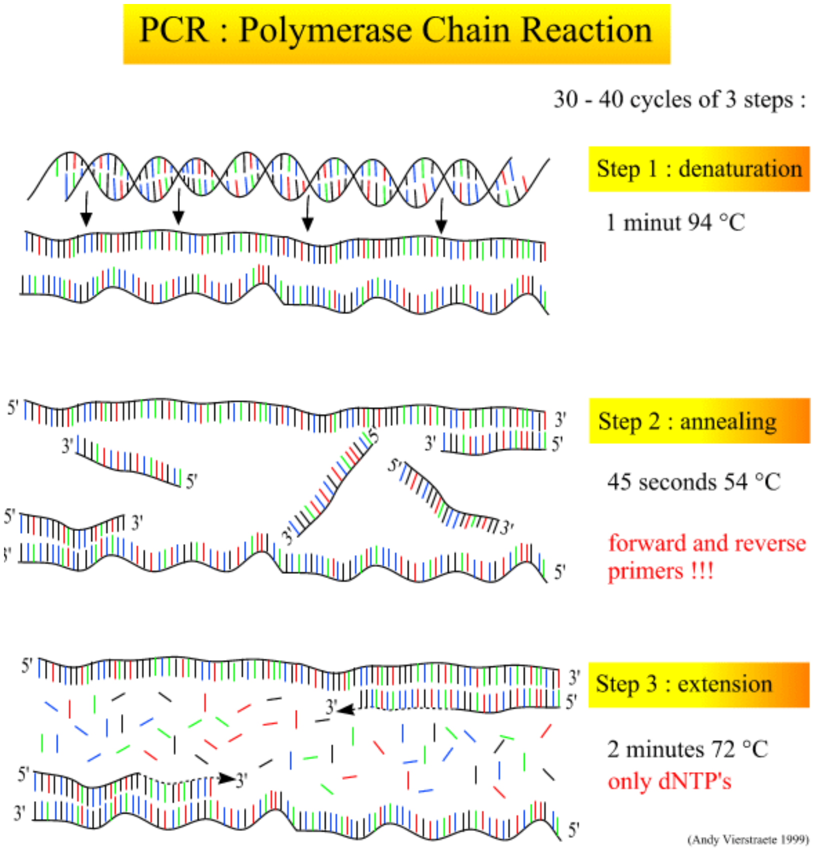


Figure 54: Polymerase Chain Reaction.

4.4.11 Polyacrylamide gel electrophoresis

4.4.11.1 Brief explanation

Electrophoretic separation of proteins and nucleic acids like DNA is performed in polyacrylamide gels. Polacrylamide gel forms by the polymerization of acrylamide monomer in the presence of small amount of N, N' - methyenebisacrylamide)Bis acrylamide). Polymerization of acrylamide requires free radical. For the formation of free radical ammonium persulphate and base TEMED (N, N, N', N' - tetramethylenediamine) is used. TEMED catalyses the decomposition of persulphate ions to give a free radical. The pore size of a gel can be varied by adjusting the concentrations of polyacrylamide and the cross-linking reagent. When a mixture of nucleic acids (Native PAGE) or proteins is applied to a gel and an electric current is applied, smaller ones migrate faster than larger ones through gel. The rate is influenced by the gel's pore size and strength of the electric field. The pores in a highly cross-linked polyacrylamide gel are quite small. Such a gel could resolve small proteins and peptides, but large ones would not be able able to move through it.in native PAGE done for separating DNA molecules instead of Sodium Dodecyl sulphate, non denaturing conditions are used ans instead of Coomassie brilliant blue, it is stained with silver nitrate. Gel Casting: Glass plates were taken and washed with detergent. The plates

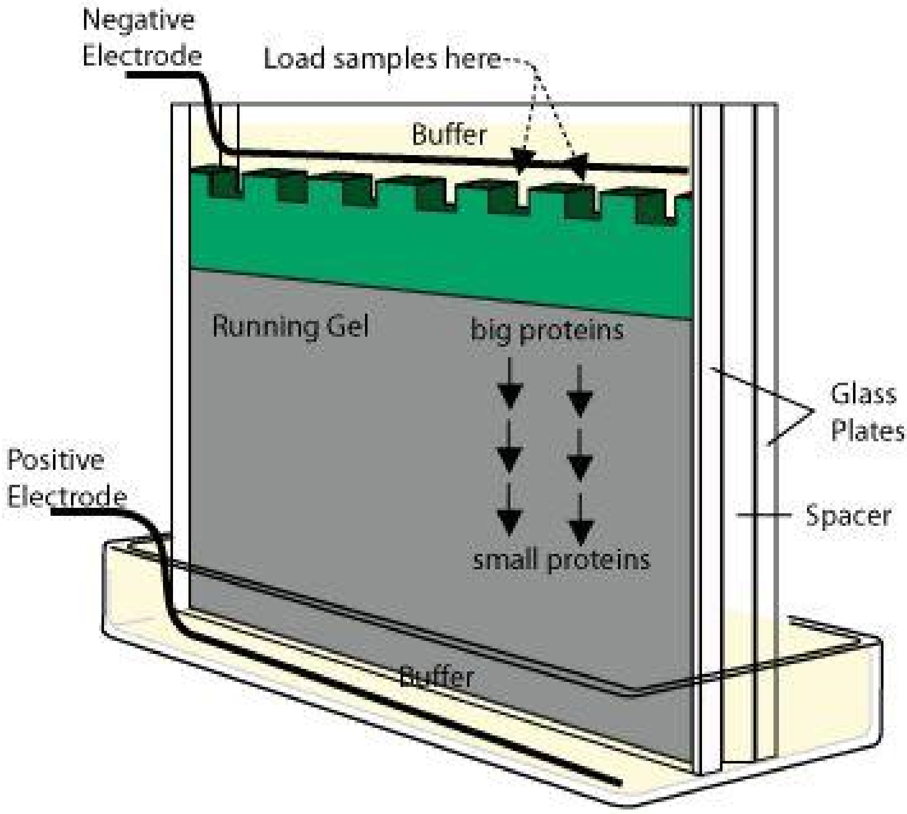


Figure 55: PAGE Apparatus.
$*$ *Source* : *matcmadison.edu/.../chapter$_5$/procedure5$_2$.htm*

were then thoroughly washed with water. They were then finally rinsed with DDW and let to air dry. The plain glass plates were cleaned with ethanol then placed on the cassette and 0.2mm spacers were placed on it. The notched plate was slid over carefully, and the arrangement was then clamped to fix the plates. Glass plates were taken and washed with detergent. The plates were then thoroughly washed with water. They were then finally rinsed with DDW and let to air dry. The plain glass plates were then cleaned with ethanol then placed on the cassette and 0.2mm spacers were placed on it. The notched plate was slid over carefully, and the arrangement was then clamped to fix the plates. This was accompanied with gentle tapping on the plates to ensure a uniform flow of the solution. Once the gel was poured comb was placed into the notch and the gel was allowed to polymerize. For finding out the length polymorphism and allele sizes of CA Repeats in different samples, a different percentage of polyacrylamide: bisacrylamide (19:1) and 10X TBE buffer as gel electrophoresis buffer was used. Run for all the amplicons was carried out at room temperature at 120 volts for 24 hrs. samples in this case were prepared by mixing 2μl of amplicons, 2μl of LP dye. Samples were then simply loaded in gel.

4.4.11.1.1 Silver staining

Following the electrophoretic separation of nucleic acids, each polyacrylamide gel was placed in a separate clean glass or plastic tray in a fixative solution and kept for 30 minutes. Then the gel was treated with 0.1% (w/v) silver nitrate solution (freshly prepared) for 30 minutes and was rinsed thrice with autoclaved water. The developer was added to the gel (1.5%NaOH+400μl formaldehyde) and treated gel developed till the bands appeared. Immediately the developer was neutralized by adding 0.75% sodium carbonate. Further, the gels were stored in 1% acetic acid.

4.4.12 Restriction fragment length polymorphism

Various restriction endonuclease enzymes recognize a particular ds DNA sequence and cut the DNA at a specific location within the recognition sequence. Any change within this recognition sequence as a result of mutation will not allow the restriction enzyme to cut the dsDNA. The vice versa can also happen i.e. a restriction site may be induced as a result of mutation. In either case there will be a change in the length of DNA fragments produced as a result of restriction by the enzyme. This is the basis of Restriction Fragment Length Polymorphism (RFLP). The PCR amplified DNA is incubated with the restriction enzyme. The presence or absence of a polymorphic site is revealed by gel electrophoresis.

Reagents for RFLP: Net reaction volume for each reaction= 9 μl

Reagent	**Stock concentration**	**Required concentration**	**Volume**
Enzyme (FOK I)	10 units/μl	1 unit	0.13 μl
Enzyme Buffer (4)	10 X	1 X	1 μl
MilliQ- WATER			3.87 μl
Target (PCR mix)			5 μl

Procedure:

1. Aliquot 5 μl of target DNA in reaction tubes or plates.
2. Prepare a master mix by taking required amounts of enzyme buffer, enzyme and autoclaved water.
3. Invert mix the master mix and centrifuge if required.
4. Add 5 μl of master mix to each of the reaction tube.
5. Mix properly and incubate at 37°C in water bath for 3 hours.
6. Analyse the restriction products on 2% agarose gel.

Part V

Results

5 Results

The present study was carried out in a total of 96 samples, out of which 48 were cases of tuberculosis and 48 were healthy controls. DNA isolation of the blood samples was carried out using phenol-chloroform method. DNA isolated from 0.5ml blood samples was diluted to 25ng/Vmul. The isolated samples were also checked for the quality.

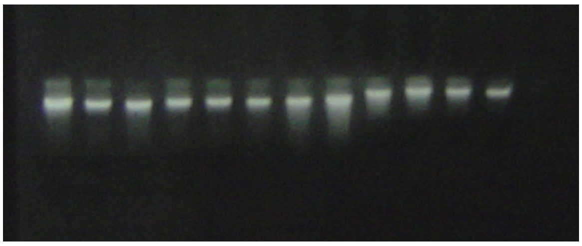


Figure 56: DNA quality-check in 0.8% agarose. 2μl of diluted DNA samples were loaded in each well.

Marker regions chosen for the study i.e, (FokI) in the VDR gene, were amplified using polymerase chain reaction and their amplification was checked on 2% agarose gel.

5.1 Results obtained for FOK I maker of VDR gene

FOK I marker of VDR gene was PCR amplified and its efficient amplification was checked on 2% agarose gel.

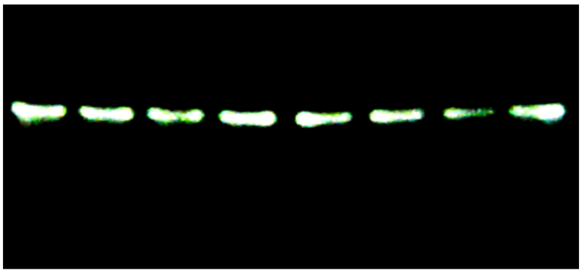


Figure 57: PCR amplified product of FOK 1 of VDR.

The fragment size obtained after PCR was of 265 bps. For further studies, the amplified product was subjected to the restriction digestion for the restriction site GGATGN with the enzyme FOK I in the wild allele. The amplified product had one restriction site for the enzyme FOK I. As a result wild allele produced 1 fragment of 265 bps, and the mutant allele produced 2 fragments of 196 bps, and 69bps. Consequently the heterozygous state produced 3 bands of 265bps, 196bps, 69bps.

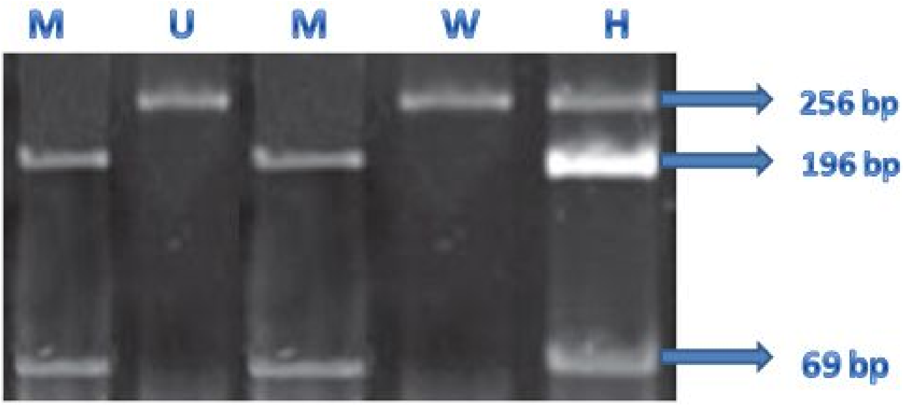


Figure 58: Representative gel of RFLP showing mutant (196bps and 69bps), Undigested (265bps), and heterozygous (265bps, 196bps and 69bps) alleles in lane 1st and 2nd and last lane of fok1 polymorphism of VDR.

The genotype obtained after performing RFLP were compared among 24 cases and 24 controls.

OBSERVED GENOTYPE	CASES	CONTROLS	TOTAL
C/C	2	10	12
C/T	22	13	35
T/T	0	1	1
Total	24	24	48

Table 12: Observed genotypes after restriction digestion in cases and control.

The allele frequency was calculated by Hardy-Weinberg Equation. The Hardy Weinberg Law states that p2 + 2pq + q2 = 1, where, p = allele frequency of allele 1, here G. q = allele frequency of allele 2, here A.

Allele Frequencies		
	CASES	CONTROLS
C	0.542	0.688
T	0.458	0.313
Total	1	1

Table 13: Comparison of the allele frequencies in cases and controls.

EXPECTED GENOTYPE	CASES	CONTROLS	TOTAL
C/C	6.5	6.5	12
C/T	17.5	17.5	35
T/T	0.5	0.5	1
Total	24	24	48

Table 14: Expected values of genotypes in cases and controls.

These observed and expected values of the genotypes are same because initially we assumed that there was no difference between observed and expected values and this was called "Null Hypothesis" moreover these observed and expected values were also used to find out the significance of the mutation in causing the disease. The significance test applied was the chi-square test.

According to chi-square (χ^2) test

$=\Sigma(Observed - Expected)^2$ / Expected

This equation was applied to calculate the chi-square value. The chi square value (χ^2) was 2.154833 and the p value obtained was 0.142.

5.2 Results for IFNγ

The present study was carried out in a total of 48 samples, out of which 24 were cases of tuberculosis and 24 were healthy controls. After DNA isolation, Marker regions chosen for the study i.e, CA repeat in the IFN γ gene, was amplified with Polymerase chain reaction and the amplification was checked on 2% agarose gel.

5.3 Results obtained for CA marker of the IFNγ gene

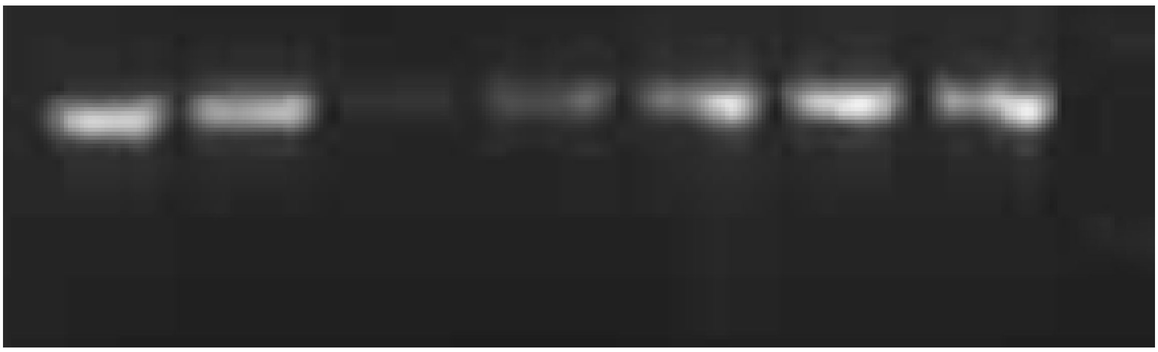


Figure 59: Amplified product of IFNγ gene on 2% agarose gel.

The size of the amplicon for the IFN γ gene was 144 bp. The samples were then checked on Polyacrylamide gel electrophoresis, with the sequenced samples as a standard marker.

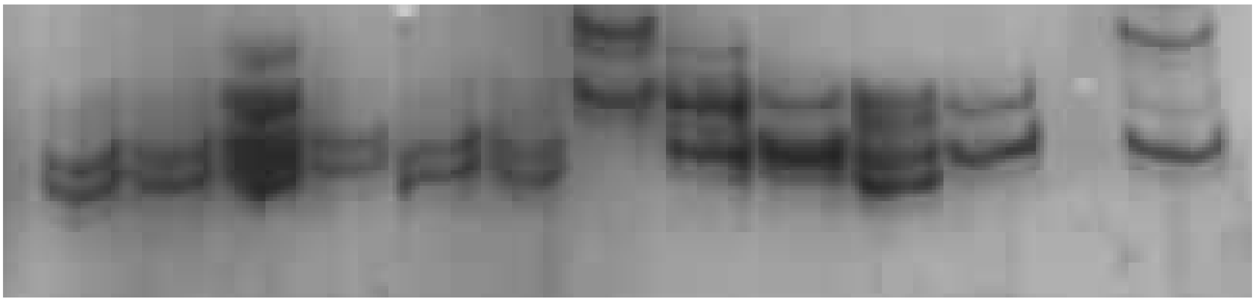


Figure 60: Representative profile of length polymorphism of Tuberculosis patients for intron 1 of the IFNγ gene using Gene Scan and Gene Tools softwares.

Similar analysis was done for all the case and control samples and data was recorded. The data obtained was as follows

Repeat number	**2n=48(%)**
12	11(22.92)
13	12(25)
14	1(2.08)
15	1(2.08)
17	0(0)

Table 15: Allele frequencies of CA repeats in healthy controls from orissa.

In healthy controls, the most frequently distributed repeat was $(CA)_{12}$ and $(CA)_{13}$, while the least were $(CA)_{14}$ and $(CA)_{15}$, the $(CA)_{17}$ repeat being completely absent.

Repeat number	**2n=48(%)**
12	14(29.16)
13	11(22.91)
14	0(0)
15	10(20.3)
17	1(2.083)

Table 16: Allele frequencies of CA Repeats in cases of Tuberculosis.

In the cases of tuberculosis from orissa, the most frequently distributed alleles of the

CA mirosatellite repeats were the $(CA)_{12}$ repeat while the$(CA)_{14}$ repeat was absent.

Repeat number	**2n=48(%)**	**2n=48(%)**
	Cases	**Controls**
12	14(29.16)	11(22.92)
13	11(22.91)	12(25)
14	0(0)	1(2.08)
15	10(20.3)	1(2.08)
17	1(2.083)	0(0)

Table 17: Frequency Comparisons between cases and controls.

Repeat number	**Controls**	**Cases**
12	11(22.92)	14(29.16)
13	12(25)	11(22.91)
14	1(2.08)	1(0)
15	1(2.08)	10(20.3)
17	0(0)	1(2.083)

Table 18: Significant Allele Frequency comparisons between cases and controls.

With GraphPad InStat software, the chi-square value for our data set was calculated. It was found to be 3.702 (degree of freedom = 1). The p-value was found to be 0.0543. The data was found not to be significant. There *was* no significant linear trend between the two categories.

Part VI

Discussion

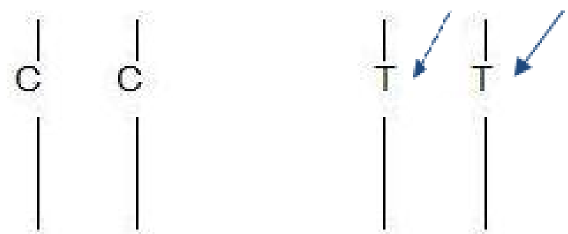
6 Discussion

Tuberculosis is an infectious disease which remains one of the largest causes of death, with 8 million new cases and 2 million deaths per annum (Dye et al., 1999). Almost one-third of the world's population is latently infected with Mycobacterium tuberculosis but majority do not develop disease, many environmental and genetic factors determine the progression of the infection. VDR is a ligand activated transcription factor, which upon binding with vitamin D3, interacts with Vtamin D response elements and signals other target genes (Malloy et al., 1999).

The studies in mouse model have shown that vitamin D-deficient mice exhibit increased sensitivity to autoimmune diseases and loss of receptor for vitamin D (VDR-deficient mice) results in oncogene- or chemo carcinogen-induced tumors (Bouillon et al., 2008). Four common polymorphisms (BsmI, ApaI, TaqI and FokI) of VDR gene have been reported, which in their different genotypic combinations, are believed to alter the immune response. FOK I is a functional polymorphism of VDR gene. Variations in the vitamin D receptor (VDR) gene have also been analyzed in different populations. It was demonstrated that the serum vitamin D deficiency may actively contribute to the increased incidence of tuberculosis among Gujarati Asians (Wilkinson et al., 2000).

The amplicon size of FOK I polymorphism of VDR is 265 bp. For further studies, the amplified product was subjected to the restriction digestion for the restriction site GAATGCN with the enzyme Fok I. Fok I polymorphism has a transition from C to T therefore accordingly the profiling was done. As a result wild allele produced 1 fragment of 265 bps, and the mutant allele produced 2 fragments of 196 bps, and 69bps. Consequently the heterozygous state produced 3 bands of 265bps, 196bps, 69bps.

If a transition from C to T occurs, then this is recognised by FOK I hence it cuts the double standed DNA.



Now, there can be three possibilities wherein the person concerned is Homozygous

dominant, Heterozygous or Homozygous recessive and accordingly we get the respective profile.

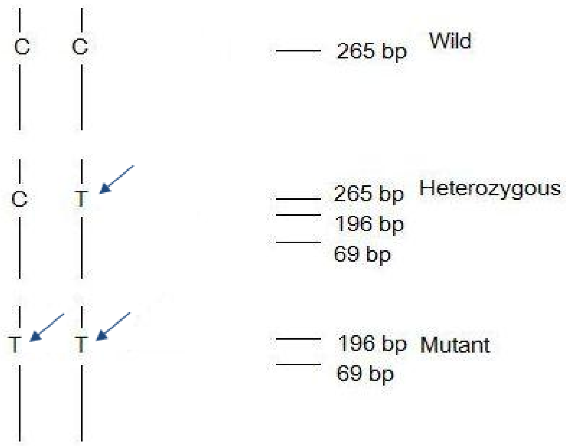


Figure 61: Profile showing homozygous dominant, heterozygous and homozygous recessive.

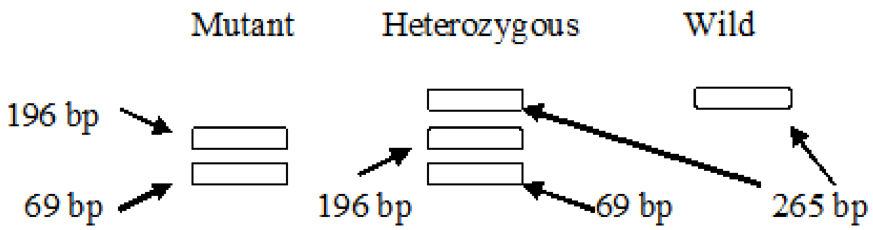


Figure 62: Schematic representation of digested products.

In our studied population of Orissa, the C allele (FOK1) was observed at a frequency of 0.542 and 0.688 in cases and controls respectively. Similarly T allele was observed at a frequency of 0.458 and 0.315 in cases and controls, respectively. The chi square χ^2 value was 2.154833 and the the p value obtained was 0.142, which was not significant. Thus, the frequency of alleles and genotypes of the fokI polymorphism of VDR did not show significant difference between cases and controls. Although, the sample size taken for present study was small to elucidate a convincing result. Thus, for exploring the relation between VDR gene polymorphisms and susceptibility to TB needs further validation using large sample size with a high power.

Tuberculosis is a multifactorial disease which results from interaction between suscep-

tible genes, environmental factors and immunological responces. IFNγ is an important Th1 cytokine, which plays a role in immune surveillance and anti tumor activity. It is a pleiotropic cytokine produced by T-lymphocytes and Natural Killer cells. Single nucleotide polymorphism and microsatellites within the first intron of the IFNγ gene correlate with a high amount of in vitro production of IFN γ and are associated with disease severity or resistance to drug therapy in various auto immune diseases. (Pravica et al, 1997). IFNγ may be associated with the development of predominant Th1-dominant, cell mediated immune responses which may enhance the expression of HLA class II antigens (Gajewski et al, 1989). Several studies have been carried out to explore the association between IFNγ in different diseases with different population groups. The $(CA)_n$ repeat within intron 1 of the IFNγ which is the functional polymorphism is associated with an increased risk to promote sporadic breast cancer (Saha et al,2005) Elevated aqueous and serum levels of IFNγ levels have been reported to be elevated in Japanese patients with Vogt-Koyanagi-Harada(VKH) disease. IFNγ plays a pivotal role in the pathogenesis of type I diabetes as it upregulates MHC class I ,II and adhesion molecules of various cells including pancreactic β cells (Campbell et al,1985) and it was also seen that transgenic mice expressing IFNγ by β cells develop autoimmune diabetes and post natal anti-IFNγ treatment prevents pancreatic inflammation in this model (Sarvetnick et al,1988); moreover the antibodies to IFNγ are found to protective against diabetes development in NOD mice and rats (Campbell et al,1991). IFNγ gene is encoded by a single gene mapped in humans to chromosome 12q24.1. A dinucleotide (CA) repeats polymorphism in the first intron of the IFNγ has been reported which has been reported to be associated with type I diabetes in Japanese as well as British Caucasoid (Ruiz et al,1993) although no association was found in Danish and Finnish patients.(Awata et al,1994)Furthermore CA repeats may also be responsible for the genetic susceptibility to endometriosis which is a gynaecological disorder characterized by pelvic pain and infertility in Japanese population (Kitawaki et al,2004).

The amplicon size of CA microsatellite repeats of IFN-γ is 144 bp. After DNA isolation, the amplified product was subjected to Native PAGE inorder to resolve the base pair differences.

As the amplicon size of CA repeats is 144 bp therefore an insertion of $(CA)_{12}$ would mean an amplicon size of 168 bp.

$(CA)_{12}$ would be 168 bp.
$(CA)_{13}$ would be 170 bp.
$(CA)_{14}$ would be 172 bp.
$(CA)_{15}$ would be 174 bp.
$(CA)_{17}$ would be 178 bp.

If both chromosomes inherited from the maternal and paternal side have insertions

of $(CA)_{12}$ Then we would get the following profile in figure 63A whereas if insertion $(CA)_{12}$ is only at one of the chromosomes then we will get a heterozygous profile as mentioned in figure 63B and if there is no insertion whatsoever then we get the profile mentioned in figure 63C.

However in multi-allelic cases insertion can be of any repeat in any possible combination. For Example there may be an insertion of $(CA)_{12}$ from the maternal side and $(CA)_{13}$ from the paternal side or just an insertion of $(CA)_{12}$ in one chromosome or an insertion of $(CA)_{13}$ in both chromosomes or an insertion of $(CA)_{13}$ in one chromosome represented by the following figure and accordingly there can be numerous combinations.

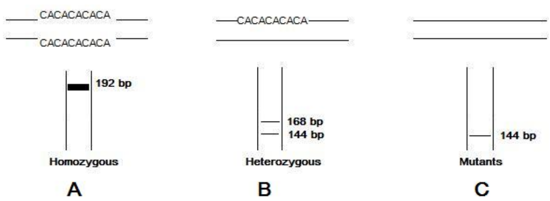


Figure 63: Analysis in Native PAGE.

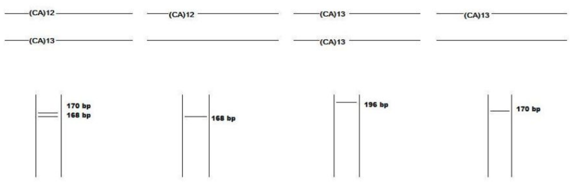


Figure 64: Multi-allelic cases of microsatellite CA repeats in IFNγ

After calculating the observed frequencies in our sample from this method, allele frequencies and expected genotype frequencies are calculated. Chi square is used to see how normal is our distribution in terms of fairness because if the number of events are more then probability of normal and fair distribution increases therefore it is beneficial to have a larger sample size for accurate and fair results. Afterwards p value is calculated to predict the significance of our results and if it is less than 0.05, then the significance is calculated from the table.

In order to dissect the role of $(CA)_n$ allelic variation and its association with susceptibility to tuberculosis, we conducted a case control study involving 24 TB patients and 24 healthy controls and obtained an increased frequency of $(CA)_{12}$ repeat (29.16) and a decreased frequency of $(CA)_{17}$ repeat (2.083) in TB patients. In healthy ethnically

matched Controls we found increased frequency of $(CA)_{13}$ repeat (25) and a same decreased frequency of $(CA)_{14}$ and $(CA)_{15}$ repeat (2.08).

The above observations, however, are preliminary indications based on the analysis of small number of samples and markers. In order to have conclusive results, an analysis with a large number of molecular markers and a large sample size will be needed.

Part VII

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